Different Strategies to Target the Oncogenic Transcription Factor EWS/FLI1 in Ewing Sarcoma

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2.1 Summary

Ewing sarcoma is an aggressive osteolytic tumour that affects children and young adults with a peak at the age of 15. It mostly affects bones such as pelvis, ribs and femur and is characterized by poorly differentiated small round blue cells.

Its main feature is the presence of a balanced translocation between chromosomes 11 and 22 leading to the generation of a dysregulated transcription factor EWS/FLI1, in which the transactivation domain of the EWSR1 gene is fused to the DNA binding domain of FLI1. This fusion protein acts as an aberrant transcription factor and mainly through regulation of its target genes drives tumorigenesis. For these reasons it represents an ideal target for treatment.

Since transcription factors lack enzymatic activities it is not possible to target them directly, and therefore new strategies need to be developed. Hence, we aimed here at identifying new and indirect ways to inhibit EWS/FLI1 activity.

From a drug screening of 153 targeted compounds we identified PI3K inhibitors and among them BEZ235 as modulators of EWS/FLI1 activity. Surprisingly, treatment with BEZ235 led to downregulation of EWS/FLI1 gene expression itself and as a consequence also of its target genes. By studying deletion constructs of the EWSR1 promote,r we identified a region of 23 bp to be important for gene expression and silencing of putative transcription factor candidates, known to be under PI3K regulation, pointed towards SP1 as candidate. Indeed, PI3K pathway inactivation reduced SP1 levels and its depletion led to EWS/FLI1 downregulation. Furthermore, we confirmed *in vitro* and *in vivo* that SP1 was indeed binding to the region identified in the promoter.

Since oncogenes are known to have superenhancer regions and since BRD4 inhibitors were described to reduce gene expression of oncogenes such as c-MYC, we decided to test JQ1, a BRD4 inhibitor, in Ewing cells. In addition to the mechanisms identified previously, we hypothesized that there might be epigenetic regulatory mechanisms involved in EWS/FLI1 expression. Hence, we pre-clinically evaluated epigenetic modifier inhibitors. Treatment with JQ1 not only reduced cell viability by induction of apoptosis but also downregulated EWS/FLI1 gene expression and its target genes, thus confirming our initial hypothesis. These results provide the first insights on the transcriptional regulation of EWS/FLI1, an area that has not been investigated so far, and offer an additional molecular explanation for the known sensitivity of ES cell lines to PI3K inhibition and to epigenetic modifiers inhibitors.

Finally, we focused on a different approach to target EWS/FLI1 activity, namely investigation of posttranslational modifications of EWS/FLI1, which were described to be important for its function such as phosphorylation. Indeed, mutations in the newly identified phospho site S287, led to a lower proliferation rate and to a reduce activation of target genes both in vitro as well as in vivo. Thus, these results indicate that posttranslational modifications such as phosphorylation are necessary for full EWS/FLI1 activity and that targeting upstream kinases could be a new therapeutic strategy.

Taken together we describe for the first time three distinct new indirect approaches to target EWS/FLI1 in this aggressive malignancy that might lead to further specific treatments alone or in combination with standard chemotherapeutic agents.

2.2 Zusammenfassung

Das Ewing-Sarkom (ES) ist ein aggressiver, osteolytischer Tumor, welcher Kinder und junge Erwachsene betrifft, wobei die Auftretenshäufigkeit um das Alter von 15 Jahren kulminiert. Der Tumor tritt hauptsächlich in Knochen des Beckens, der Rippen und im Oberschenkel auf und besteht aus undifferenzierten, „kleinen runden blauen“ Zellen.

Das Hauptmerkmal des ES ist das Vorhandensein einer balancierten Translokation zwischen den Chromosomen 11 und 22, die zur Fusion der zwei Transkriptionsfaktorgene EWSR1 und FLI1 führt. Das resultierende Fusionsprotein EWS/FLI1 enthält die Transaktivierungsdomäne von EWSR1 und die DNA-Bindungsdomäne von FLI1. Das Fusionsprotein agiert als aberranter Transkriptionsfaktor und ist durch Beeinflussung der Expression einer Reihe von Zielgenen an der Tumorigenese beteiligt. Es stellt daher auch ein ideales Ziel für eine ES-Behandlung dar.

Da Transkriptionsfaktoren keine enzymatische Aktivität mit entsprechender Struktur enthalten, ist es schwierig, sie mittels kleimolekularer Inhibitoren zu blockieren. Es müssen daher alternative Strategien zur Inhibition entwickelt werden. Das Ziel der hier beschriebenen Arbeit ist die Identifizierung von solchen neuen Ansätzen zur Blockierung der Aktivität von EWS/FLI1.

Dazu haben wir als erstes den Einfluss von 153 zielgerichteten Inhibitoren auf die Aktivität von EWS/FLI1 getestet. Dabei wurden verschiedene PI3K-Inhibitoren inklusive BEZ235 als effiziente Blockierer der EWS/FLI1-Aktivität identifiziert. Es zeigte sich, dass die Behandlung von ES Zellen mit BEZ235 zu einer Reduktion der Expression von EWS/FLI1 und als Folge auch seiner Zielgene führt. Durch Analyse des EWSR1-Promotors mittels Deletionskonstrukten konnten wir eine 23 bp lange Region identifizieren, welche eine wichtige Rolle bei der Expression des Fusionsproteins spielt. Durch genetisches Ausschalten von verschiedenen Transkriptionsfaktoren, welche in dieser Region binden könnten und nachweislich durch PI3K reguliert werden, wurde SP1 als potentiell involvierter Kandidat identifiziert. Wir konnten anschliessend bestätigen, dass Inaktivierung des PI3K-Signalweges zur Reduktion der SP1-Proteinexpression führt, und auch, dass Ausschalten von SP1 die Menge an exprimiertem EWS/FLI1 reduziert. Ausserdem konnten wir bestätigen, dass SP1 sowohl in vitro als auch in vivo an die identifizierte Region im EWSR1-Promoter bindet.

Es ist bekannt, dass die Expression von Onkogene oft durch so genannte „Superenhancer“-Regionen reguliert wird. Wir haben daher spekuliert, dass dies auch bei EWS/FLI1 der Fall sein könnte und dass involvierte epigenetische regulatorische Mechanismen die Expression von EWS/FLI1 steuern. Ein wichtiger epigenetischer Regulator in „Superenhancer“-Regionen ist das Protein BRD4, gegen das es verschiedene Inhibitoren gibt. Ein Beispiel ist das Molekül JQ1, welches die Expression von verschiedenen Onkogenen wie c-Myc reduziert. Wir haben den Effekt von JQ1 in ES Zellen getestet und konnten zeigen, dass JQ1 nicht nur die Lebensfähigkeit der Zellen beienflusst, indem es in den Zellen Apoptose induziert, sondern auch, dass es die Expression von EWS/FLI1 und seinen Zielgenen runterreguliert. Somit wurde unsere initiale Hypothese bestätigt.

Diese Erkenntnisse sind die ersten, welche einen Einblick in die transkriptionelle Regulation von EWS/FLI1 geben, welche bis jetzt noch nicht untersucht wurde. Die Daten liefern auch eine Erklärung für die bekannte Sensitivität von ES Zellen gegenüber Inhibitoren des PI3K-Signalweges und epigenetischen Regulatoren.

Als einen weiteren möglichen Ansatz zur Inhibierung der EWS/FLI1 Aktivität haben wir abschliessend nach posttranslationalen Modifikationen des Fusionsproteins gesucht, welche seine Funktion beeinflussen. Dabei haben wir Ser287 als Phosphorylierungsstelle identifiziert und konnten zeigen, dass diese Stelle die Aktivität von EWS/FLI1 beeinflusst. So reduziert Mutation von Ser287 die Expression von EWS/FLI1-Zielgenen und die Proliferationsrate von ES-Zellen in vitro und in vivo in einem Xenograft-Modell. Dies zeigt, dass postranslationale Modifikationen wie Phosphorylierungen für die volle Aktivität von EWS/FLI1 nötig sind und dass Inhibition der beteiligten Kinasen eine mögliche therapeutische Strategie darstellen könnte.

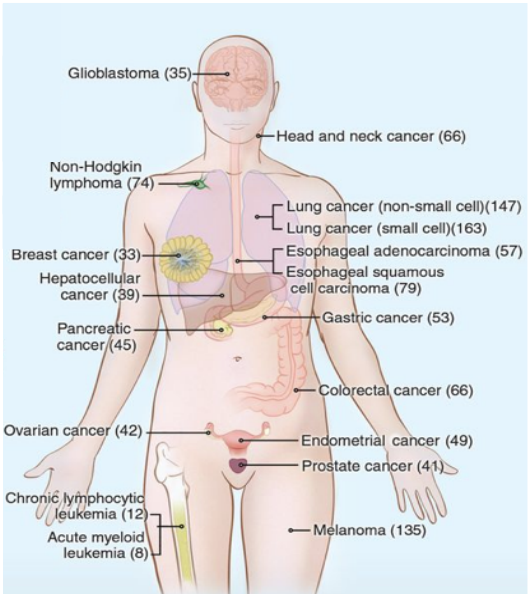
Zusammenfassend zeigen unsere Daten drei neue Möglichkeiten für die indirekte Blockierung der Aktivität von EWS/FLI1 im ES auf. Diese könnten alleine oder in Kombination mit Standard-Chemotherapeutika zu einer Verbesserung der Therapie des ES führen.

3.Introduction

3.1 Cancer

Cancer is a pathology characterized by uncontrolled growth of abnormal cells that, if not stopped, can lead to death [1]. A tumour can be induced by external factors like tobacco and UV lights or internal ones such as genetic alterations, meaning different mutations that alter and promote expression of oncogenes and silence tumour suppressors [2]. In adults, in a common solid tumour there are about 50 gene mutations, 95% of which are characterized by single-base substitutions [3] and 90% of these single mutations are missense mutations, followed by nonsense mutations and others affecting splicing sites or untranslated regions. In tumours like melanoma or lung cancer there are up to 200 different mutations that have been described where paediatric cancers in general have a much lower mutational burden (Fig. 1A). Mutations can occur in all chromosomes with different rates and types (Fig. 2A) and include deletions, amplifications, swapped or inverted sequences and single-base mutations and also alterations on the level of chromosomes such as translocations [4, 5].

A



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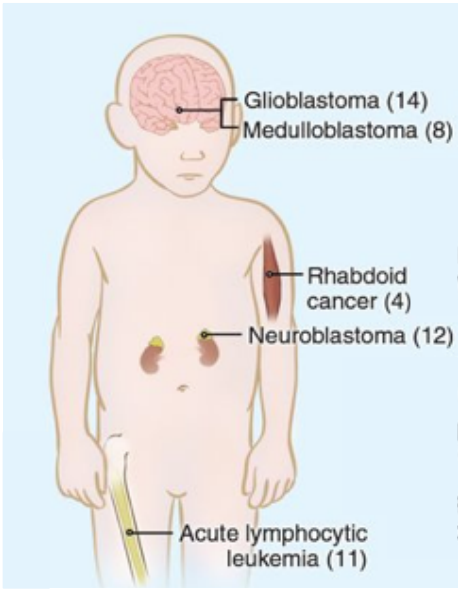

Fig. 1 Variety of cancer in adults and its genome.
Different types of tumours present in adults (A) and children (B); numbers in brackets represent the median value of nonsynonymous mutations identified by genome-wide sequencing studies (Modified by [3]).

A tumour arises after acquiring a series of mutations. This process has been described for the first time in colorectal cancer where after a first mutation called *gatekeeping* [6] that allows a selective growth advantage of the cell, new mutations occur [7, 8]. Not all the acquired mutations confer advantages to the cells and if so they are defined as *passengers*. D*river* mutations instead give cells a further growth advantage [5] and usually represent alterations in those genes involved in important regulatory circuits or pathways (Fig. 2B).

The number of alterations in a tumour cell is also correlated with age, especially in some cancers derived from self-renewing tissues [9]. In these cases, initial mutations happen already in a stage of preneoplasia, namely in a precursor cell. This is true for glioblastomas and pancreatic cancers in adults, and for leukaemia in children [10]. It might also explain why younger patients have a lower mutation rate (Fig. 1B).

A B

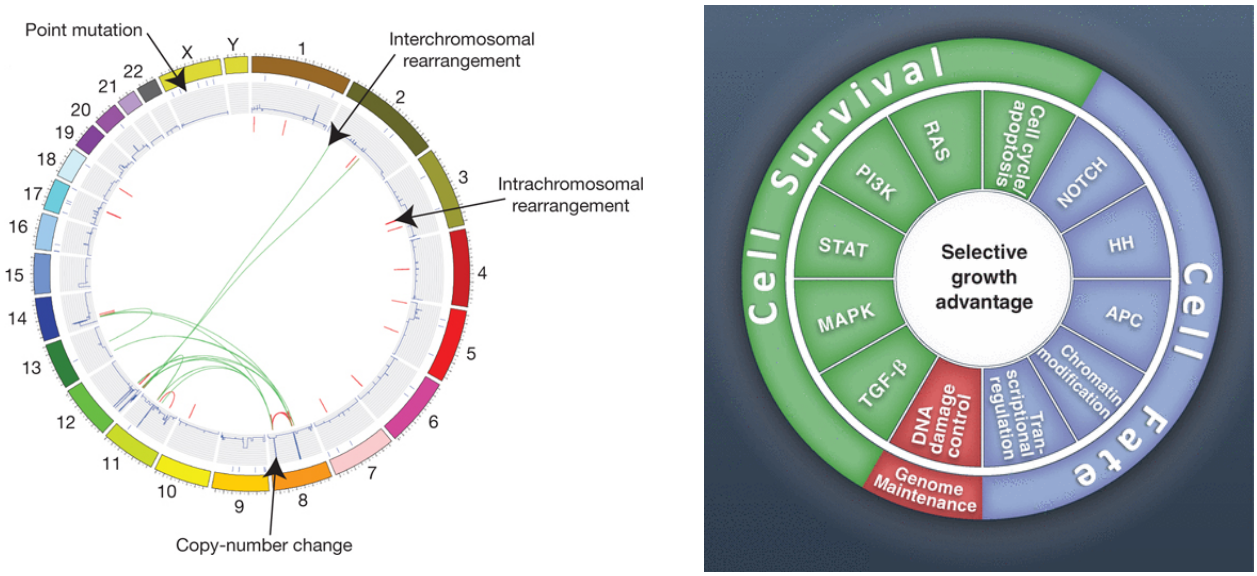

Fig. 2 Variety of genomic alterations and their role in tumorigenesis.

(A) Landscape of somatic mutations [4]. (B) Driver mutations occur in genes involved in cell survival, cell fate or genome maintenance [3].

To define a tumour, six main requirements have to be fulfilled which are better defined as hallmarks of cancer and which are acquired during tumour development [11]. A tumour cell is self-sufficient in proliferative signals, meaning that it can promote and sustain autonomously cell divisions by overcoming negative feedback loops in proliferative pathways [12, 13], or acquiring mutations [12-14] or also overexpressing genes involved in such signalling [15-17], or by promoting ligand production in stroma cells [18, 19]. Resistance to growth suppressors, meaning insensitivity to antigrowth signals is another hallmark. In normal conditions, inhibitory signals provoke either a G_0 arrest in cell cycle or cells will differentiate. Also in this case, mutations of tumour suppressor genes as Retinoblastoma (RB) [20, 21] or overexpression of oncogenes as c-MYC [22-24] provoke a malignant phenotype. A second hallmark is that cells are capable of unlimited replications, in fact they do not undergo senescence after a limited number of divisions [25] but they continue to replicate and become immortal [26]. Another aspect of such cells is that they are resistant to apoptosis, they do not respond anymore to stimuli that induce programmed cell death. The most common mutation that leads to apoptosis resistance is in the p53 gene which is also called the genome guardian and is found mutated in >50% of cancers [27]. But also overexpression of survival genes is responsible for similar effects [28-31]. Another aspect to take into consideration is that tumours in order to survive need nutrients and oxygen, therefore new vessels are required through a process called neoangiogenesis [32-34]. This process is driven by Vascular Endothelial Growth Factor (VEGF) which is secreted by tumour and stroma cells. It promotes new vessel formation and alteration of pre-existing ones [35, 36]. Finally tissue invasion and metastasis formation are the most dangerous acquired capabilities of a tumour and they are responsible for 90% of death [37], indeed some cells from the primary tumour can invade other tissues and form metastasis.

To these hallmarks, the same authors added further properties such as changes in metabolism and resistance to immune system, adding genome instability and promotion of inflammation as enabling conditions at the basis for all hallmarks [38] (Fig.3). Indeed it has been shown that cancer cells can reprogram their energy metabolism and even in presence of oxygen they can save energy and do glycolysis [39-41].

The second emerging hallmark was recognized due to the fact that immunocompromised patients are more likely to develop tumours [42] and this has also been verified in mice, proving that the immune system plays a role against cancer [43, 44].

Knowledge about tumour cell behaviour, capabilities and underlying mutations offers many different options for developing a specific targeted therapy.

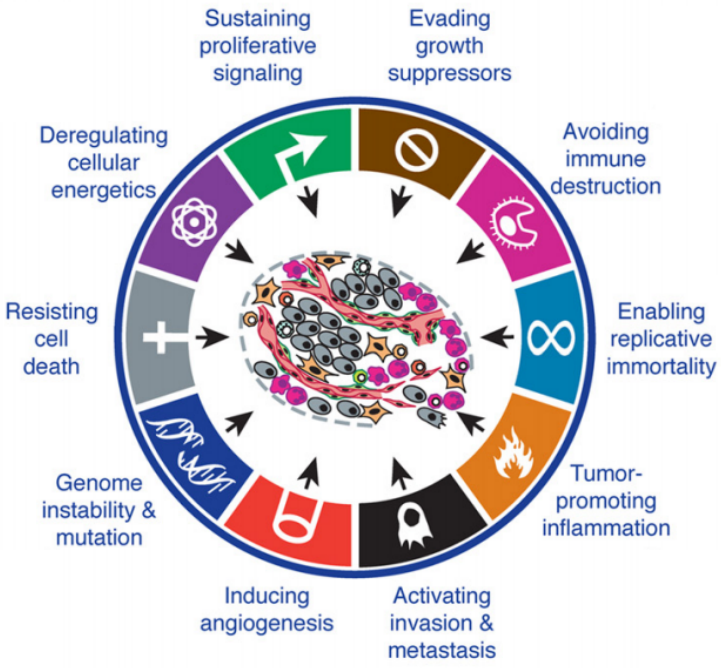

Fig. 3 Hallmarks of cancer [38].

3.2 Paediatric tumours

Paediatric tumours constitute only 2% of all cancers [45] and have an incidence of 175'000 cases per year with a mortality rate of 96'000. Most of childhood cancers arise sporadically and present somatic mutations in tumour suppressors or proto-oncogenes; in some cases tumours are also the result of a chromosomal translocation. They affect kids and young adolescence with an age of 19 and lower and represent the second leading cause of death in this population [46, 47]. Nevertheless thanks to molecular genetics, over the past 30 years, patients have an increased prognosis with an overall survival of 80% (Fig. 4A). Despite this 5 year survival rate, patients need to be monitored regularly in order to recognize any second malignancies, chronic diseases or functional impairments early [48]. Over the years, the incidence rate of some tumours compared to others has increased (Fig. 4B) and this might correlate with the fact that environmental changes have occurred or that today it is easier to diagnose such kind of tumours at an early stage.

According to patient's age, the frequency of tumours differs. In patients with an age between 0 and 14 years, the most frequent childhood tumour is Acute Lymphocytic Leukaemia (ALL) with an incidence of 30% followed by brain tumours (Fig. 4C), whereas in adolescence patients the most frequent type of cancer is Lymphoma [49].

Another factor that seems to influence tumour distribution is the gender, because males are slightly more affected. Finally the geographic area of the patient might be important since high incidence of cancers is registered in developing countries [50-53].

Patients affected by paediatric tumours are diagnosed with a certain delay because the initial symptoms are very similar to the ones of any other common diseases [54]. They present fever or illness, loss of energy, localized pain, loss of weight, headache, pale skin, weakness, and nausea. Treatment of these tumours does not differ from adult's ones, indeed chemotherapy, radiotherapy and surgery are also used in paediatric cancer treatments alone or in combination. Despite the fact that survival rates are increasing, there are still many side effects due to standard treatments, therefore targeted therapy could be a solution.

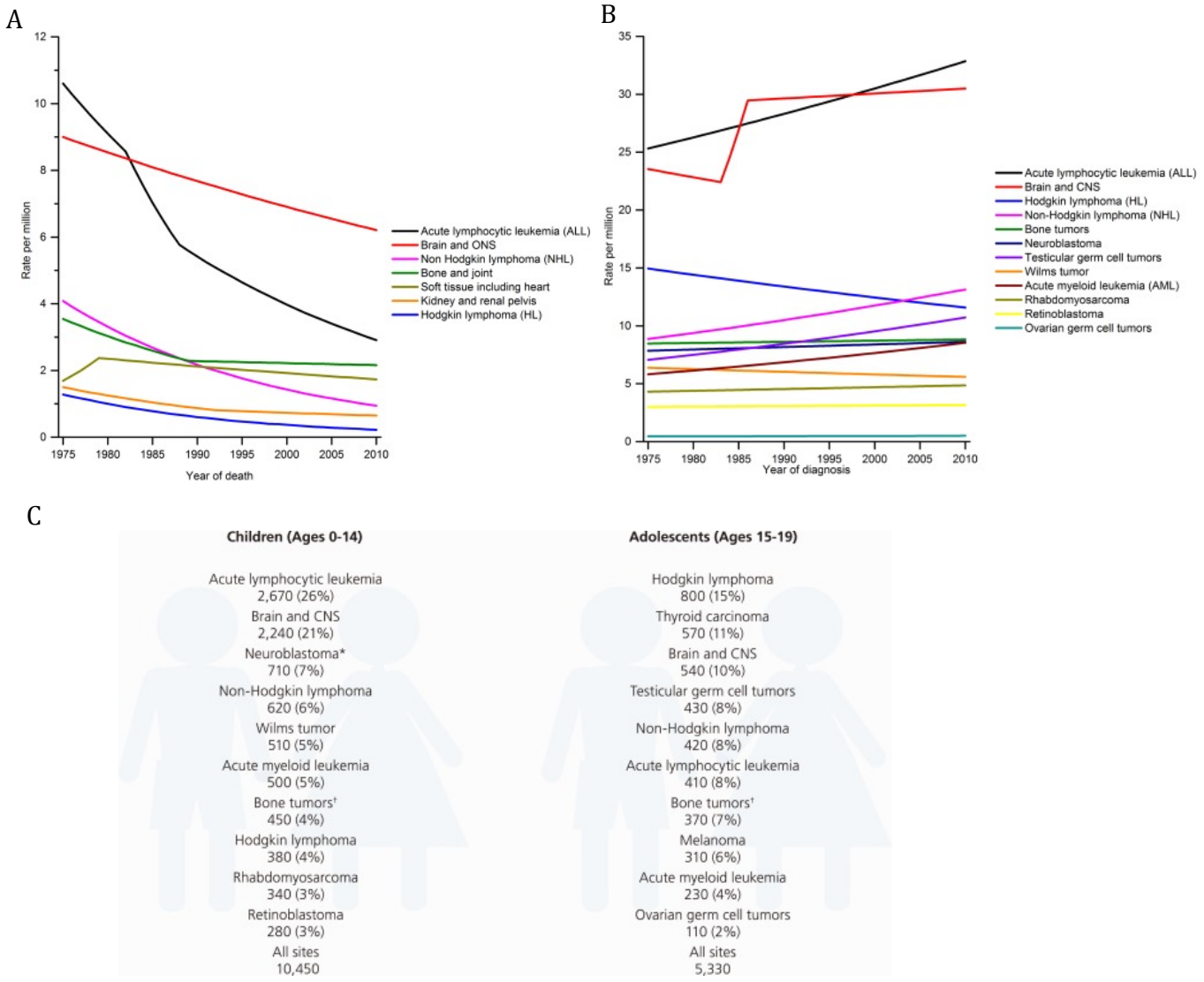

Fig. 4 Types of childhood cancers.
(A) Mortality rate from 1975 till 2010. (B) Cancer incidence rate by site from 1975 till 2010. (C) Frequency of different types of cancer in two subgroups of paediatric patients. Modified from [49].

3.3 Targeted cancer therapy

Targeted therapy is a specific form of treatment that targets specifically cancer cells based on molecular differences compared to normal cells. Normal chemotherapeutics have a cytotoxic effect on cells whereas the targeted therapy has more a cytostatic effect, so that cells cannot proliferate anymore.

Cancer cells can either overexpress specific markers that can be directly targeted by the use of a specific antibody, or, when there are functional differences from normal cells, the therapy can target those upon which cells are dependent on [55]. In Table 1 the most well known approved targeted compounds are shown and divided according to the different type of targeting dependencies: genetic, lineage, host and synergy (not shown).

A genetic dependence links a genetic alteration to tumour formation and the first case has been described in the 60s' when a chromosomal translocation was found to be responsible for chronic myeloid leukaemia (CML). The new chromosome, named Philadelphia chromosome, presents a fusion gene BCR-ABL where the Abelson Murine leukaemia viral oncogene (ABL), encoding for a protein kinase, is under the regulation of Breakpoint cluster region (BCR). Targeting specifically this mutation by blocking the ABL kinase activity with a tyrosine kinase inhibitor, Gleevec, led to 80% response rate [56]. Following the same path, Herceptin, an antibody blocking Epidermal Growth Factor Receptor 2 (ERBB2), found amplified in 20% of breast cancers, was developed for treatment [57].

Table 1 | Targets of approved cancer drugs

Cancer drug	Target	Disease indication	Genetic validation	
			DNA changes	Protein expression
Genetic dependence				
Gleevec	BCR–ABL	CML, ALL	Translocation	Disregulated protein is expressed
Herceptin	ERBB2	Breast cancer	Amplification, increased copy number	Overexpressed
Lineage dependence				
Tamoxifen	Oestrogen receptor	ER+ breast cancer		Overexpressed
Host dependence				
Avastin	VEGF receptor	Colon cancer, pancreatic cancer		Overexpressed

Table n.1 Targeted Therapy approved drugs. Modified from [55]

The synergy dependence is based on the fact that two mutations can be synthetic lethal in a tumour cell and not in a normal one. A clear example is the case of breast cancer when in presence of a mutation in BRCA1, treatment with Poly (ADP-ribose) Polymerase (PARP) inhibitors results in cancer cell death [58]. The treatment mimics a loss of function mutation in PARP and this second mutation in combination with the first one lead to apoptosis.

Lineage dependence refers instead to the fact that cancer cells and normal cells, from which the tumour derives, are both dependent on the same pathways and this is the case for some breast cancers [59] and prostate cancer [60].

In case of host dependence, the target is the microenvironment of a tumour. For example some tumours like colorectal cancer are dependent on neo vasculature and blocking VEGF with either a specific antibody named Avastin or small molecule inhibitors targeting VEGF receptor activity, results in some efficacy in treatment [61]. Targeted therapy is used in many cancer treatments nowadays and knowing possible targets for each tumour opens a new variety of possibilities for a better treatment and for limiting side effects.

3.4 Ewing sarcoma

Ewing sarcoma (ES) is a rare paediatric bone and soft tissue tumour that affects kids and young adults, mostly males (1.5/1) with a peak at the age of 15 (Fig.5A). The incidence is very low, 3-4 people per million every year are diagnosed with ES [62-65]. Besides this low rate it represents the second most common bone tumour in kids, second only to osteosarcoma and accounts for 3% of all paediatric malignancies [66]. The majority of patients are white Caucasians, whereas in Africans or Asians the disease is very uncommon suggesting a genetic contribution [65, 67, 68].

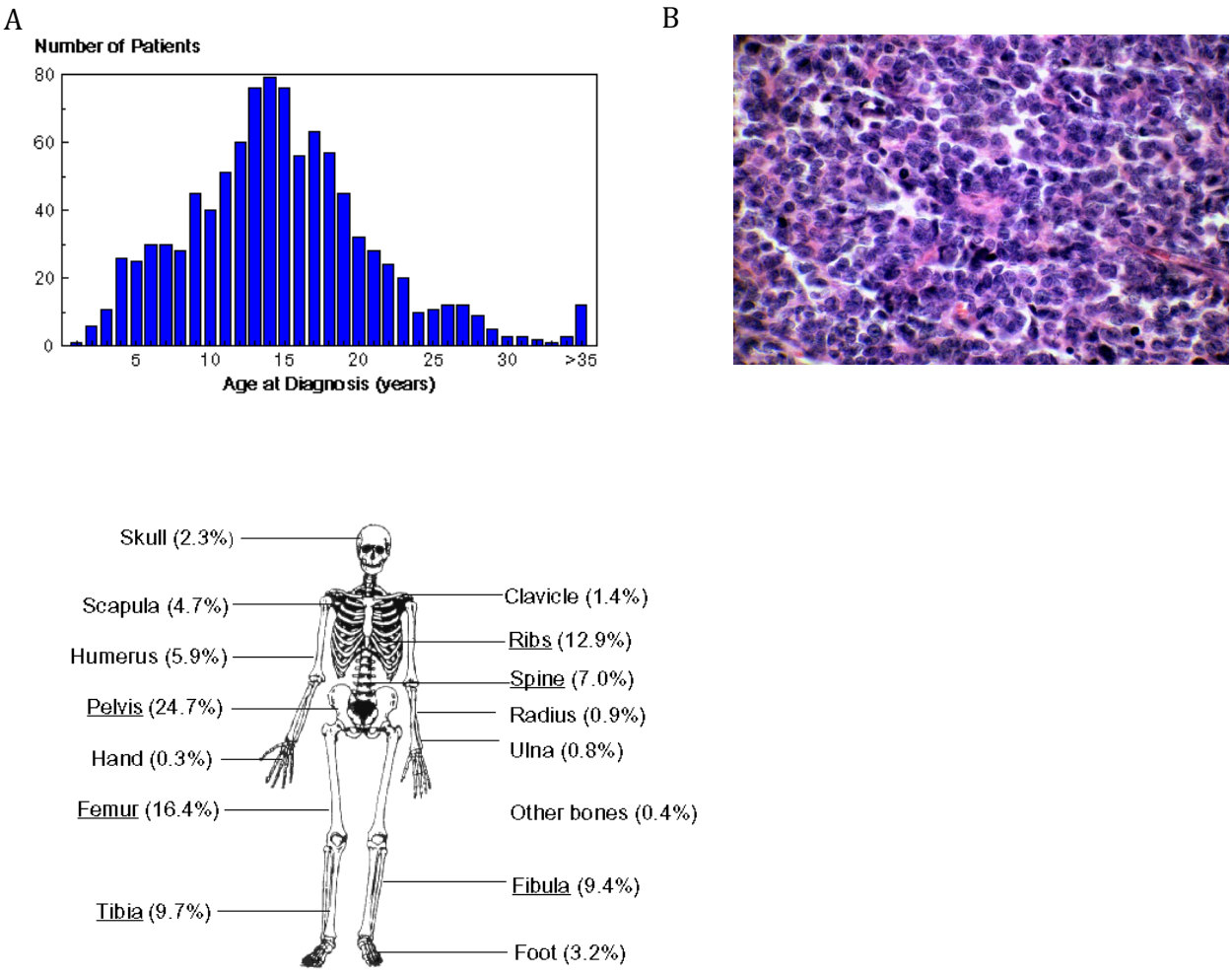

Fig. 5 Ewing Sarcoma.
(A) Age of ES patient (from [69]). (B) H&E staining of Ewing sarcoma cells (from [69]). (C) Sites affected by ES, numbers represent percentages (from [70]).

Both osseous and extra-osseous Ewing sarcoma together with peripheral primitive neuroectodermal tumours (PNET) and Askin tumour are part of the Ewing's sarcoma family of tumours (ESFT), which is characterized histologically by undifferentiated small round blue cells (Fig.5B), high nucleus/cytosol ratio, high mitotic rate, small single nucleoli, stained positive for cell membrane glycoprotein CD99 (MIC2) [71]. On the molecular level, they are characterized by the presence of a balanced translocation between members of the TET family of RNA-binding proteins and E-Twenty-Six or erithroblastosis-virus-associated transforming sequences (ETS) family of transcription factors [71, 72]. This translocation gives rise to an oncogenic fusion protein that is thought to drive the tumorigenesis.

Ewing sarcoma, previously known as round cell sarcoma of bone, takes its name from James Ewing, the pathologist that first characterized it in 1921 [73]. He described it as a diffuse endothelioma and later as a endothelial myeloma with high radio-sensitivity [74]. At that time, defining a tumour based on morphology was a novel concept, whereas today we know that the main feature in Ewing sarcoma is the presence of a balanced translocation between chromosome 11 and 22,t(11;22)(q24;q12), leading to the generation of a dysregulated transcription factor EWS/FLI1 in 85% of cases [75].

3.4.1 Prognosis factors

Patients affected with ES present pain and swelling at the site of tumour. After biopsy, immunohistochemical examinations together with molecular techniques (Fluorescent in situ hybridization, FISH) are needed for a correct diagnosis. The outcome of ES patients is affected by different prognostic factors [76-78]. The site of the tumour is one of these: EF mostly arises in bones such as pelvis, ribs and femur (Fig. 5C). Patients have the best prognosis when the disease is localized at the extremity of bones, when it is proximal/central in the bones or affects the pelvis site, then outcome is dismal [76-78]. Another factor that influences the outcome is the gender and the age of the patient, in fact a female kid younger than 15 years has a better prognosis compared to patients that are older and males [78]. ES arises both in bones and soft tissues with different frequencies and it appears that the location of the tumour changes with the age of the patient. In 20% of cases the tumour presents in the pelvis, whereas 50% are found in the extremity of bones, a scenario usually found in a younger patient.

Adults with ES present the tumour in soft tissues more frequently than kids [67]. Another factor that might affect the ES outcome is the fusion protein type (Fig. 6). Type I patients have a better outcome compared to the type II tumours, according to *in vitro* studies that described type I as having a less effective transactivation domain and lower proliferation capability [79-81]. The same correlation has been described also *in vivo* using a murine model [82]. Also the genomic alterations present in the tumour, in addition to the fusion protein, are associated with an unfavourable outcome [83, 84]. Further the tumour size largely contributes to the outcome, together with the presence of which are found in 25% of cases at diagnosis. Patients with metastasis in the bones or bone marrow have a poorer prognosis (<25%) compared to those that present lung metastasis (50%). ES indeed has an early tendency to metastasize and the 5 year survival rate from 70% for those with localized tumour decreases to 20-30% in patients with metastasis [85-89].

3.4.2 TET family: EWSR1

EWSR1 on chromosome 22 is a ubiquitously expressed RNA binding protein and its function has not been fully characterized and it is fused in ESFT also to other ETS members such as ERG (10%), ETV1, ETV5 and FEV but with a lower frequency [90, 91]. Together with translocated in liposarcoma (TLS) and TATA-binding protein associated factor 15 (TAF15), it belongs to the TET family of proteins (FIG. 6) [92]. These TET proteins are involved in several processes like splicing, DNA repair and transcription [92], they are localized in the nucleus and ubiquitously expressed in children and adults [93, 94]. They were first described as fusions with ETS family members in cancer, and present at the amino terminus a conserved transactivation domain that drives and enhances the transcriptional activity of the ETS transcription factors in the fusion proteins [93, 95]. This amino terminus region of EWSR1 is highly disordered [96, 97], whereas the carboxy terminus contains a RNA binding domain and it is well structured [98]. The breakpoint region of TET proteins has been described to encode a site that recognize DNA binding proteins, Topoisomerase II [99] and extra nucleotides that might promote DNA double strand break [100].

3.4.3 ETS family: FLI1

FLI1 is a member of the ETS transcription factor family, characterized by a DNA binding domain helix-turn-helix at the carboxy terminus [101, 102]. The ETS family contains FLI1, ERG, ETV-1 and E1AF, they bind DNA in purine rich sequences with a GGAA/T core at the promoter of several genes [103-106].

ETS transcription factors regulate either positively or negatively the expression of genes involved in differentiation, development, transformation, haematopoiesis, metastasis, cell proliferation, apoptosis and angiogenesis [102].

The same ETS domain that is important for the interaction with DNA is also responsible for protein-protein interaction and a single aminoacid mutation can alter the binding [107, 108]. FLI1 is involved in the development of the hematopoietic and vascular system [109] and in adults its expression is strictly regulated and restricted to hematopoietic cells [110].

3.4.4 EWS/FLI1

EWS/FLI1 is a fusion protein containing the amino domain of EWSR1 (Ewing sarcoma break region 1) protein and the carboxy domain of FLI1 (Friend Leukemia Integration 1), a transcription factor member of the ETS family (Fig. 6) [111].

According to the site of rupture/chromosomal breakpoint, the fusion gene derived thereof is classified as type I, II or III (Fig. 6). EWS/FLI1 type I is the most common one (60% of cases), where exon 7 of EWSR1 is fused to exon 6 of FLI1 (7-6). Type II (25% of cases) instead has one exon more from the FLI1 gene (7-5) whereas the type III is the most infrequent with the first 10 exons from EWSR1 fused to exon 6 of FLI1 (10-6) [112]. It is thought that the RNA Binding Domain (RBD) of TET protein might alter the oncogenic function of EWS/FLI1, that would explain why it is not present in the fusion protein [113] and the same consideration can be applied also for the weak FLI1 transactivation domain displaced by the one of EWSR1.

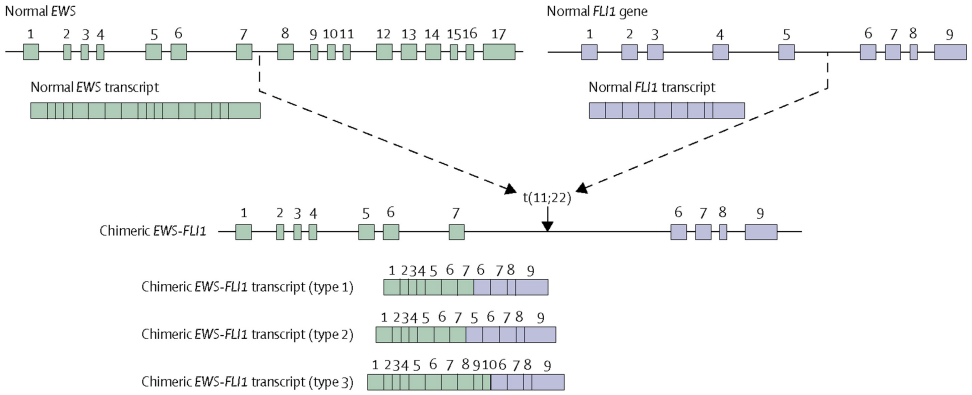

Fig.6 EWS/FLI1 fusion proteins.[114].

EWS/FLI1 fusion protein recognizes, at least *in vitro*, the same binding sites as FLI1 [103], but having a stronger transactivation domain compared to the wild type (wt) protein. It is largely considered that the oncogenic potential derives from the deregulated expression of its target genes [115, 116]. It has been shown also that the activation of target genes is directly proportionated to the number of GGAA repeats at the promoter level and in fact EWS/FLI1 binds both at ETS binding site and to GGAA/T microsatellite sequences and this has been demonstrated also *in vivo* [117]. Even if the DNA binding domain of the fusion protein derives from FLI1, surprisingly the signature of ES samples differs from the hematopoietic tissue expressing wt FLI1, indicating that the DNA binding domain is not per se driving the malignancy [118-120].

As most of paediatric tumours, Ewing sarcoma does not contain numerous alterations in addition to the translocation [3, 121]. In 2014 whole genome sequencing performed by two different laboratories identified few but consistent mutations in several tumour patient samples [66, 122]. Although EWS/FLI1 translocation has been described in all samples, recurrent mutations in tumour suppressor genes as Stromal Antigen 2 (STAG2), P53 and Cyclin-Dependent Kinase Inhibitor 2 (CDKN2A), gain copy number in chromosome 3 and 8 and loss of STAG2 were observed together with other small alterations (Fig. 7). Particular attention has been paid to STAG2 loss, since it correlates with a worse prognosis and is associated with recurrent/metastatic samples.

Nevertheless in the majority of patients it has been shown that the fusion protein is the only mutation [3, 121-126] and ES oncogenesis is believed to originate from this single genetic event. EWS/FLI1 has been proven to be sufficient and necessary for tumour initiation and progression [66, 127] and regulates the expression of a variety of target genes acting as aberrant transcription factor [128]. Depletion of EWS/FLI1 leads to an arrest in cell growth and cell death via apoptosis [129] *in vitro* [130-135] and a reduced tumour growth *in vivo* [136]. For these reasons EWS/FLI1 represents an attractive target for therapy [137].

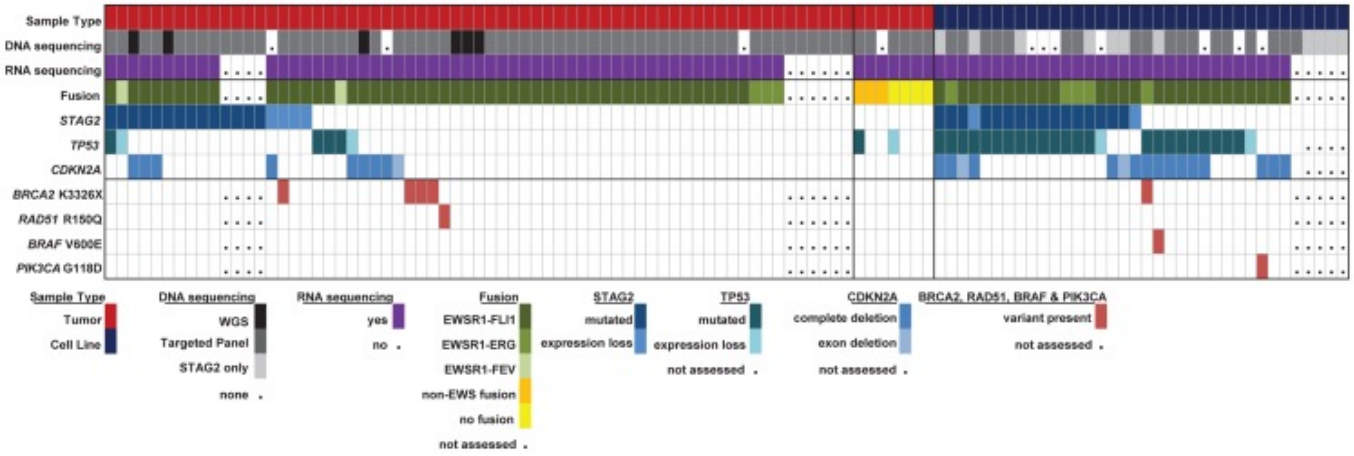

Fig.7 *Sequencing results of tumour samples-in red- and cell lines- in blue.*
Schematic representation of most frequent alteration present in EFT (modified from [66]).

Being a transcription factor, EWS/FLI1 is considered to be undruggable [138]. In fact transcription factors lack enzymatic activities and due to intrinsically disorder domains, crystallization remains challenging and so does the design of a targeted compound. EWS/FLI1 indeed has been described as intrinsically disordered protein (IDP) [97, 139], having as structured region only the FLI1 DNA binding domain [139, 140]. Such a non structured region confers to the oncofusion protein the capability to escape from degradation which misfolded proteins would incur into [141]. For these reasons it is also difficult to narrow down hypothetical protein interaction surfaces and therefore also to identify hypothetical targets.

3.4.5 EWS/FLI1 function: Target genes

EWS/FLI1 expression has been shown to be required for maintenance of transformation [118, 142-144] and to drive tumorigenesis through regulation of its target genes [145-147].

Gain of function studies in heterologous cell systems and silencing of the fusion protein in Ewing cells, revealed that EWS/FLI1 is both involved in their upregulation as well in repression [118, 143, 144] due to direct interaction with their promoter or with other regulatory elements such as enhancers. To do that, the fusion protein activates or represses gene expression of downstream targets interacting with either co-activators such as CBP/p300 [148] or co-repressors such as the NuRD complex and Histone deacetylase LSD1 [149, 150].

Among the target genes no physiological driver oncogene has been identified yet, suggesting that EWS/FLI1 per se is the main oncogenic driver mutation. Nevertheless some targets have been described to be important for the oncogenic potential and to be involved in several processes such as cellular senescence [151], evasion of apoptosis [149, 152] and regulation of cell cycle [153]. Indeed some of them are transcription factors or protein modifiers and their activation or repression targets other downstream genes, amplifying in this way the oncogenic effect.

Recent studies described a correlation between the distance of EWS/FLI1 binding site from the transcriptional start site [117, 154, 155] and it seems that when the transcription factor is closed to the start site, the target gene will be activated, on the other hand, binding sites located further upstream are correlated with repression of gene expression [155]. This is just a first indication, but it is also generally thought that the DNA sequence of the promoter is important for activation or repression upon EWS/FLI1 binding.

Besides the transcriptional regulation function, others have not all been characterized. Indications suggest that depending on different protein-protein interaction EWS/FLI1 can have different activities. A full list of possible interactors has been published, some of them belong to spliceosome complexes [140].

Hence, the fusion protein might not only act as transcription factor [156, 157], but it seems that the fusion protein builds a network hub involved in the regulation of alternative splicing [158, 159]. It is thought that this is an additional mechanism used by the oncoprotein to drive and maintain the malignancy.

3.4.6 Activated target genes

Among the activated target genes, the most relevant ones for transformation are: nuclear receptor subfamily 0 group B member 1-NROB1 [142, 160], c-MYC [115], VEGF [161] Insulin-like growth factor 1 (IGF1) [162], NK2 homeobox 2 (NKX2.2) [144] and cyclin D1 (CCND1) [163]. In addition, EWS/FLI1 positively regulates gene expression of Glioma associated oncogene 1 (GLI1) transcription factor [164] which is a downstream effector of the Sonic Hedgehog (Shh) pathway found active, upregulated or amplified in different types of cancers. Nevertheless the Shh pathway in Ewing sarcoma seems not to be active, so expression of GLI1 is not sufficient to switch on signalling and drive tumorigenesis.

Among the activated ones, NKX2.2 and NROB1 cannot be directly targeted, but their depletion reduced oncogenic capacity.

NKX2.2, a transcriptional repressor involved in neural differentiation and never associated with tumorigenesis [165], has been identified as important for oncogenic transformation. Upon knockdown of NKX2.2, whose target genes overlap with the ones of EWS/FLI1, the transformation is lost both *in vitro* and *in vivo* [143].

However, silencing of the fusion protein and simultaneous overexpression of NKX2.2 failed to rescue the tumorigenic potential. Also NROB1 or DAX1, a nuclear receptor and a directly activated target gene of EWS/FLI1, has been indicated as important for tumorigenesis (Fig. 8A). Indeed silencing of NROB1 induces G1 arrest, impairs cell proliferation, inhibits cell anchorage independent cell growth and blocks proliferation *in vivo* in xenograft model [142, 160, 166]. Another activated target gene that when depleted impairs tumour proliferation and blocks cell anchorage independent growth is GLI1 [167, 168]. In addition it has been proven that among GLI1 target genes there is also NKX2.2 [164], demonstrating once again the intrinsic and complex network behind this disease (Fig. 8B) [169].

3.4.7 Repressed target genes

Among the repressed target genes, pleckstrin homology-like domain, family a, member 1 (PHLDA1) [170], Insulin-like growth factor-binding protein 3 (IGFBP3) [118], lysyl oxidase (LOX) [150], Forkhead Box O1 (FOXO1) [171], Transforming Growth Factor Beta Receptor 2 (TGFBR2) [172] have been described. The mechanism by which EWS/FLI1 regulates its repressed target genes appears to be very complex involving not only transcriptional regulation, but also post transcriptional, epigenetic modulation [156], repression of activators [171] and activation of transcriptional repressors [144, 173-175]. However, due to Chromatin immuno precipitation (ChIP) studies, it is now possible to identify the target genes directly activated or repressed by the fusion protein and further investigate their role in the pathology of the disease [117, 154, 155, 176].

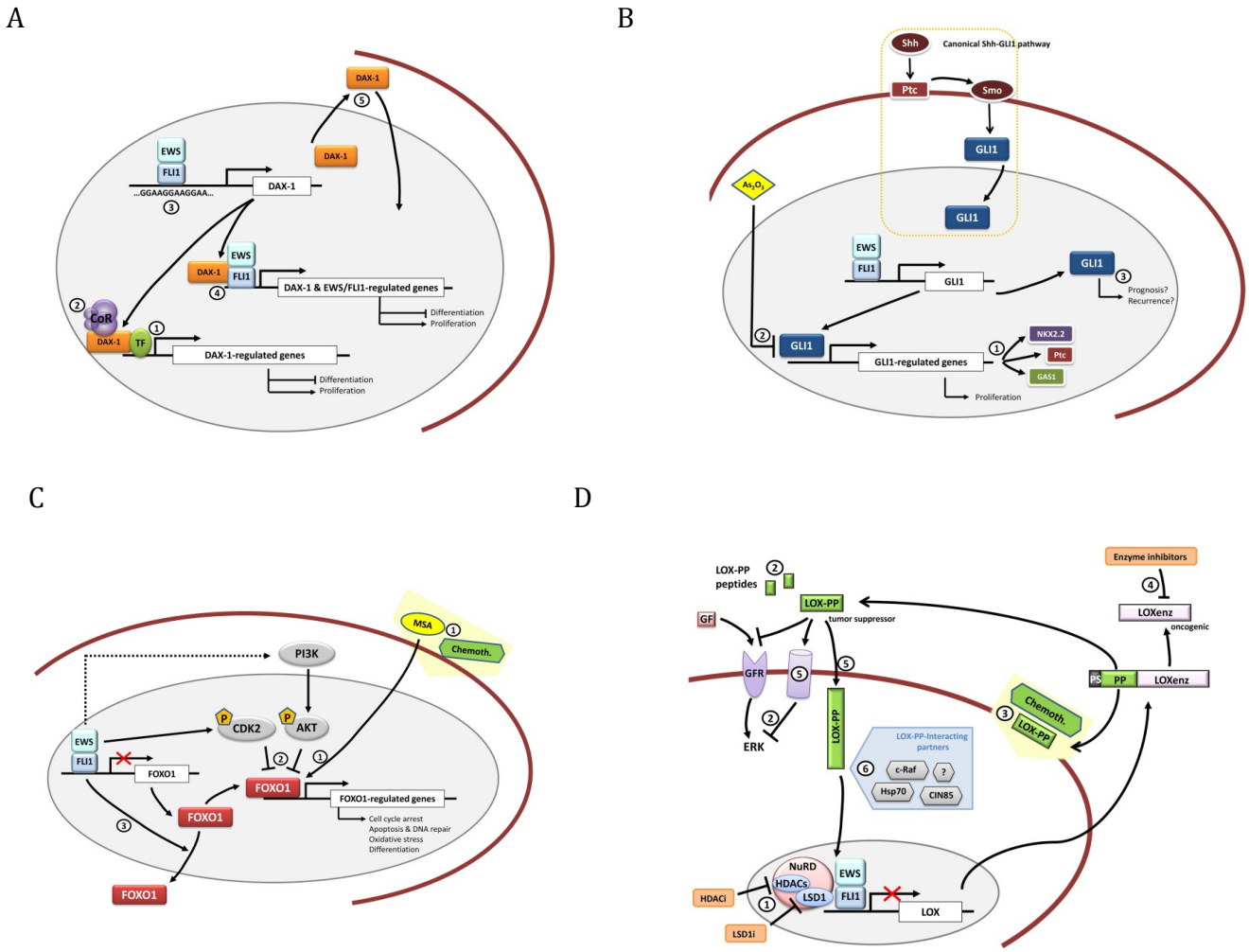

Fig.8 *Role of EWS/FLI1 target genes*
Function of activated target genes NROB1 (A) and GLI1 (B); and repressed target genes FOXO1 (C) and LOX (D). Modified from [169]

It has been shown that downregulation of FOXO1 [171, 177] and LOX [150, 178] is required for tumorigenesis (Fig.8 C and D).

FOXO1 is a transcriptional regulator involved in cell proliferation and survival which blocks cell cycle by inducing a G1 arrest [179], promotes cell death and DNA repair [180]. When downregulated, it induces angiogenesis [181, 182]. In Ewing sarcoma EWS/FLI1 represses FOXO1 at different levels, namely alteration of its localization and transcription, both impairing its function. Overexpression of FOXO1 inhibits cell proliferation confirming FOXO1 as tumour suppressor gene in ES. For targeting ES, methylseleninic acid (MSA) a compound that enhances and reactivates FOXO1 activity has been tested [183].

Indeed treatment with MSA induces cell death *in vitro* and *in vivo*, but it might also induce some toxic effects. Nevertheless in lower doses it can be suggested as a therapy in combination with standard treatments.

The LOX gene encodes for lysyl oxidase which is an enzyme involved in the maintenance and integrity of the extracellular matrix [184-186]. The protein is secreted in an inactive form and then processed once outside the cell. The LOX-PP fragment is the one responsible for tumour suppression whereas the other fragment constitutes the active form of the enzyme that when expressed at high levels is tumorigenic [187]. Therefore tight regulation of LOX is required in the cells to maintain a normal physiological status.

It has been shown that LOX functions as tumour suppressor. Its depletion led to oncogenic phenotype in fibroblast [188] and it has been found down regulated in several cancer types [189-193]. Being important for tumour suppression, induction of its expression would be indicated as therapeutic approach. However, overexpression of full length LOX might not be optimal, since together with the LOX-PP there is also the enzyme to be overexpressed [194]. To circumvent this problem, induction of expression of the LOX-PP fragment alone in Ewing cells blocks cell proliferation, cell anchorage independent growth and cell migration possibly due to the inhibition of PI3K/AKT, ERK, NFKB pathways *in vitro* and *in vivo*. Surprisingly also expression of the full length LOX in Ewing impairs tumour formation meaning that tumour suppressor component prevails [178]. In other cancer cells instead, expression of complete LOX induces tumorigenesis [178, 195, 196]. Due to the tumour suppressor function of LOX-PP, a recombinant fragment has been investigated as therapeutics in different cancer types and showed reduced tumour cell growth also in combination with other chemotherapeutics such as Doxorubicin [187, 191, 194, 197-200].

More studies are needed to investigate the tumour suppressor role of LOX-PP fragment and its interactors in Ewing sarcoma, but it is evident that its regulation is necessary for tumorigenesis and tumour maintenance.

3.5 Therapy

Current therapy is a combination of chemotherapy, surgery and radiotherapy, but none of them alone is sufficient to eradicate the tumour. As already described by James Ewing, ES has a high sensitivity to radiotherapy, nevertheless this alone was only responsible for 10% survival [201], the same rate obtained when non complete surgery was performed. In addition to this, radiotherapy treatments could lead to secondary diseases and impair bone formation, so different approaches were needed.

To improve the survival rate, in the early 1960s first treatments using chemotherapeutic agents were performed, namely treatments with cyclophosphamide (C) alone [202, 203], then in combination with vincristine (V) and radiotherapy [204] which led to an increase in survival up to 24%.

Later, Actinomycin D (A) and Doxorubicin (D) were added to the current ES treatment [205] further increasing the 5 year survival rate up to 60%. To the pre-existing VACD regime, Etoposide and Ifosfamide have been added [87] and the same combination of 4-6 drugs is still in use nowadays.

After diagnosis of ES, the usual step is an initial cycle of chemotherapy to reduce the tumour mass followed by radiation and/or surgery to completely eradicate the disease [206]. Adults are treated in the same way as kids, skeletal tumour as extraskeletal ones and local tumours as the metastatic lesions [67].

Despite the fact that the survival rate of ES patient increased from 10% to 70% thanks to this combination of chemotherapeutic agents together with surgery and radiotherapy, there is still a poor survival rate for those who present with metastasis. Unfortunately, there is no specific therapy so far for refractory or recurrent ES. In addition to that, the ES therapy in use today is still similar to 40 years ago and so far no new drugs have significantly improved survival, and toxicity and morbidity induced by such treatment is relatively high [207-210]. Therefore new targeted approaches are needed to improve outcome especially for metastatic and recurrent or refractory Ewing's patients [211].

3.6 Novel approaches

Current Ewing sarcoma therapy is very toxic and not specific. Due also to recurrences and metastasis there is an urgent need for the development of new therapeutic approaches.

Besides the usual chemotherapeutic agents VACD used for standard treatment, retinoic agent was under study as possible new drug. Albeit it showed promising results in different cancers [212-214], in the case of ES it was not very effective [215, 216].

Fenretinide, a synthetic retinamide, was shown to have antitumor activity where retinoic acid failed [217-219], and to induce apoptosis mediated by ROS (reactive oxygen species) through phosphorylation of p38 MAPK [220]. Three years later the same group described that the effect of fenretinide is lost when EWS/FLI1 is depleted, suggesting a high specificity of the drug for this tumour [221]. Indeed fenretinide upregulates death receptors and cotreatment with dead receptor ligands resulted in a synergistic effect and increased cell death [222]. This compound is currently in phase II clinical trial for solid tumour treatment. Despites these promising data, researchers have focused their attention to identify alternative specific approaches.

Since EWS/FLI1 is the master regulator of tumorigenesis, it represents the main attractive target for therapy. Indeed the fusion protein has been defined as the perfect target without a therapeutic agent [137]. Upon depletion of the fusion protein apoptosis occurs. Ewing sarcoma A673 cell line instead was used to further investigate the role of EWS/FLI1 in the cells, because it has been shown that A673 cells are not affected in cell growth or in cell cycle progression by EWS/FLI1 knock down [143]. As a result it appears that the presence of the fusion protein is responsible for changes in cellular morphology, adhesion and migration [223]. Upon depletion of the fusion protein, cells loose their capability to adhere to tissue culture plastic, migrate less and actin cytoskeletal integrity is increased. This indicates that Ewing cells and therefore the fusion protein itself is responsible for mesenchymal cell features.

In other Ewing cell lines, silencing of EWS/FLI1 with either siRNA or antisense oligonucleotides decreases cell proliferation and viability leading cells to death via apoptosis *in vitro* and tumour regression *in vivo* [127, 133-135, 224-226]. Indeed EWS/FLI1 regulates cell cycle progression by targeting directly or indirectly some components such as p21/CDKN1A [148], Cyclin D1 [157, 227], Cyclin E [228], TGFbeta [229], p57/KIP2 [153], IGF1 [118, 230] or MAPK [231].

Also the effect of EWS/FLI1 depletion on apoptosis could be explained by the direct interaction of the fusion protein with Casp3 [232] or by the indirect regulation of TNF [233] and again affecting IGF1 [118, 230] and TGFbeta [229].

In general it seems that EWS/FLI1 is required to maintain tumour growth by many and different mechanisms. Knowing more about these could lead investigators to new targets for a therapeutic intervention.

3.6.1 Targeting downstream of EWS/FLI1

Since EWS/FLI1 functions as transcription factor and its target genes are involved in the oncogenic transformation, the first approaches for a targeted therapy focussed on downstream targets to mimic a block of EWS/FLI1.

Silencing or targeting the most oncogenic target genes of EWS/FLI1 with specific compounds led to impressive results, comparable to the silencing of the fusion protein itself [143, 234].

Among the activated EWS/FLI1 target genes some can be directly targeted.

VEGF is responsible for neoangiogenesis and therefore tumour maintenance through vessel formation. Combination treatments of a VEGF antibody, Avastin, together with standard chemotherapeutics induces partial remission [235, 236], even though the contribution of the single VEGF inhibitor is not clear.

Protein Kinase C Beta (PKRCB), instead, encodes for a protein kinase that is directly regulated by the fusion protein. It is involved in cell survival and tumour maintenance *in vivo*. Silencing by siRNA or inhibition with specific compounds such as Enzastaurin induces apoptosis and prevents tumour growth *in vivo* [234]. Despites these promising results, Enzastaurin treatments have different outcomes according to the type of cancer [237-240]. Nevertheless Ewing cells are among the most sensitive ones and PKC-β inhibition resembles the effects of EWS/FLI1 depletion, hence more insights into the mode of action are needed.

In Ewing sarcoma, some components of the IGF1 pathway have been identified as directly regulated target genes of EWS/FLI1. IGF signalling is implicated in malignant transformation and tumour resistance in different type of tumours [241, 242] and compounds targeting the tyrosine kinase receptor function or antibodies blocking IGF1 binding have been developed and are currently in phase I and II studies [243-245].

EWS/FLI1 represses IGFBP3 gene expression, a repression necessary and important for ES development [246, 247], and enhances expression of IGF1 ligand [248, 249] and of its receptor IGF1R [250]. Further, it regulates Caveolin expression important for IGF1R internalization [251] and downregulates several microRNAs that target this pathway [252]. Overexpression of the ligand which is responsible for the pathway activation and maintenance, creates a feedback loop that represents an attractive target for therapy [230, 253]. The binding of IGF1 to its trans membrane tyrosine kinase receptor activates downstream PI3K, RAS-MAPK and JAK-STAT pathways that promote cell proliferation and survival [254-257]. Blockage of IGF1 signalling by targeting IGF1 ligand with specific antibody [258], its receptor IGF1R by treatment with a tyrosine kinase inhibitor [259], or antisense technology [260] induces cell death *in vitro* and *in vivo* [230, 258, 259, 261]. In addition inhibition of IGF1 signalling sensitizes Ewing cells to standard chemotherapeutics [262, 263]. Indeed also the IGF1R small molecule inhibitor ADW742 in combination with normal chemotherapeutic agents such as Doxorubicin, Vincristine and Imatinib resulted in synergistic effects on Ewing cell lines [264].

The repressed target gene IGFBP3, responsible for IGF1 sequestration, has been found down regulated in a panel of Ewing cell lines indicating the IGF1 signal as constitutively activated and administration of exogenous IGFBP3 to the cells blocked Ewing cells growth and alter cells adhesion property [79, 265]. Thus while IGFBP3 can be considered a potential anticancer molecule in ES [118], its use might lead to osteoporosis and other undesired toxic effects.

Unfortunately treatment with IGF1R antibody, such as figitumumab or R1507 and AMG479, in mice and during first clinical trials led to rapid development of resistance [261, 266-270]. So far the mechanisms of resistance are not fully understood but there are some recent indications that IGF1R cells switch signalling from IGF1/IGF1R to IGF2/IR-A [271, 272]. To overcome the resistance to IGF1R inhibition, targeting IGF1R downstream pathways like ERK, MAPK, AKT/mTOR which are constitutively active in ES [231, 273, 274] might improve outcome. Inhibition of these pathways with specific drugs led to reduction of cell growth and survival upon G1 arrest in Ewing cells [231]. Indeed, treatment with Rapamycin, a specific inhibitor for mTOR [275], reduced EWS/FLI1 levels in Ewing cells [276] and when combined with IGF1R resulted in a better outcome *in vitro* and *in vivo* and also patients with refractory disease could benefit [264, 274, 277-280].

mTOR (target of Rapamycin) is a serine/threonine kinase involved in cell growth and metabolism and forms two complexes (Fig. 9). Rapamycin is specific for mTOR complex 1 and was discovered in 1970s from *Streptomyces hygroscopius* in Rapa Nui island [281].

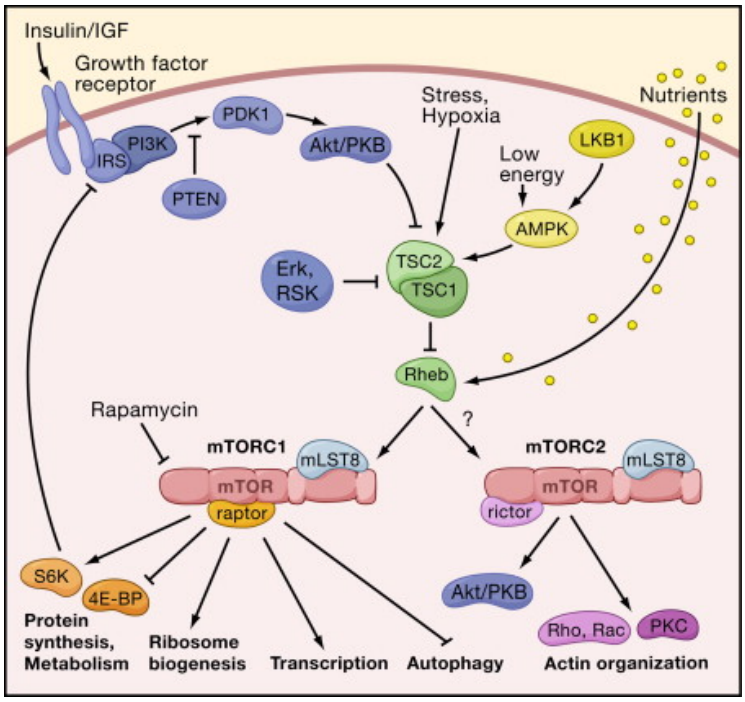

Fig. 9 *mTOR signalling*. Modified from [281]

However, also resistance to Rapamycin occurs pretty quickly due to crosstalk between AKT activation and the IGF1R pathway [282-284]. Indeed sarcoma patients treated with mTOR inhibitors such as Rapamycin and its derivatives showed promising results [285]. Nevertheless, some of these patients expressed high levels of activated AKT [283, 286] indicating that blocking mTOR is a way to stop sarcoma growth, but its inhibition alone is not enough to prevent recurrences. Following the same idea, investigators have focused on targeting PI3K signalling at different levels downstream of IGF1. This pathway, often activated in cancers, is indeed involved in tumorigenesis, progression, invasiveness and formation of metastasis [287]. Therefore, a large number of drugs to inhibit different components of the pathway have been developed [288] (Table n.2).

Group	Selectivity	Compound	Cancer type	Genetic determinant of response	Clinical trial status
I Pan-class I	Class I PI3K	GDC-0941 (Roche/Genentech)	Breast	HER-2 amplification PIK3CA mutations	I-II in breast, non-Hodgkin's lymphoma, NSCLC
			Melanomas, MM, non-Hodgkin's lymphoma, NSCLC, ovarian	–	
		BKM120 (Novartis)	Breast	PIK3CA mutations	I-II in breast CRC, endometrial, GIST, GBM, leukemia, melanoma, NSCLC, pancreatic, renal cell, SCCHN, TCC
			CRC, endometrial, GIST, GBM, leukemia, melanoma, NSCLC, pancreatic prostate	–	
		PX866 (Oncothyreon)	Breast, CRC, MM, NSCLC, pancreatic prostate, ovarian	PIK3CA mutation, PTEN loss	I-II in CRC, GBM, NSCLC, SCCHN
		BAY 80-6946 (Bayer)	Advanced solid cancers	PIK3CA mutations	I-II in advanced solid cancers
II Isoform specific	PI3Kα	GDC-0032 (Roche/Genentech)	Solid cancers	–	I-II in solid cancers
	PI3Kβ	GSK2636771 (GlaxoSmithKline)	Advanced solid cancers	–	I-IIa in advanced solid cancers with PTEN deficiency
	PI3Kγ and PI3Kδ	IPI-145 (Infinity)	Hematological malignancies	–	I-IIa in advanced hematological malignancies
	PI3Kδ	CAL-101 (Gilead Sciences)	AML, CLL, Hodgkin's and non-Hodgkin's lymphoma, MCL	–	I-II-III in AML, CLL, Hodgkin's and non-Hodgkin's lymphoma, MCL, MM
III Dual PI3K/mTOR	PI3K and mTOR	BEZ235 (Novartis)	Breast	PIK3C2a mutation, HER-2 amplification, PTEN loss	I-II in breast, renal cell
			Ovarian	PIK3C2a mutation, PTEN loss	

Table n.2 *PI3K inhibitors classes. From [288]*

Several studies have focused on the dual inhibitor BEZ235 in ES. This compound was described to inhibit growth of melanomas [289], breast cancers [290], gliomas [291], and of Ewing cells [292]. Treatment with BEZ235 induces a G1 arrest leading to a lower proliferation rate, blocks cell migration, reduces adhesion of Ewing cells and delays tumour growth in xenograft model [292].

3.6.2 Small molecules screening

Unfortunately targeting directly downstream targets of the fusion protein by single agents did not lead to significant results, therefore new approaches were developed. Small molecule screenings have been conducted to identify new drugs capable of blocking the activity of EWS/FLI1 [170, 293, 294]. The idea behind this is to link gene expression profile modulation to small molecules treatments to design a more specific therapy [295].

The first drug screening performed on Ewing cells, based on gene expression profiles, identified AraC (cytosine arabinoside) as a compound capable of attenuate the EWS/FLI1 gene signature [293]. AraC was described as a molecule with strong activity against ALL [296, 297]. The molecule, having a structure similar to cytosine, binds to the DNA hence interfering with DNA synthesis and therefore inducing cancer cells death. Fusion protein target gene expression was decreased upon AraC treatment due to a reduction in EWS/FLI1 protein levels. Thus, AraC led to a decrease in cell viability and transformation, reduced anchorage independent growth and induced cell death *in vitro* and *in vivo*, therefore abrogating tumour growth in a xenograft model. Despite these first promising pre-clinical results, hemato-toxic effects were observed in a phase II study and there was no strong reduction in tumour burden in patients with refractory or relapse ES [298].

In a similar screening Mithramycin an antibiotic already under study for ES treatment [299, 300] was identified. This compound binds to GC rich region in the genome at the promoter level of different genes including SRC, MYC and MDR1, competing with SP1 and other transcription factors for the binding [301-309]. Indeed treatments with Mithramycin affect Ewing cells, leading to DNA damage, PARP inactivation and surprisingly have a direct effect on EWS/FLI1 activity, decreasing expression of activated target genes [294]. It has been shown that the drug suppresses tumour growth in different Ewing xenografts. Since Mithramycin does not affect EWS/FLI1 level, but only its activity, this would suggest it as inhibitor acting downstream of the fusion protein. Mithramycin is currently in phase I/II clinical trial and also more potent and specific analogues are being tested [310].

More recently, again from a screening, kinase inhibitor Midostaurin (PKC412) has been indicated as modulator of EWS/FLI1 activity [170]. Treatment of A673 cells with 1280 chemical compounds identified 21 drugs capable of reducing 20% NROB1 and EWS/FLI1 gene expression and

upregulating at least 50% the repressed target gene PHLDA1. A second screening was performed adding three more cell lines and two additional target genes as read out for EWS/FLI1 activity, NKX2.2 and CAV1. From this second screening, besides the chemotherapeutic agents used in the clinic, the broad kinase inhibitor Midostaurin was identified.
This compound was previously shown to have activity against sarcoma [311]. PKC412 treatment of Ewing cell lines led to a reduction of EWS/FLI1 activated target genes and overexpression of PHLDA1, normally repressed by the fusion protein. Neither EWS/FLI1 expression nor localization was affected by the treatment, so the drug is not modulating the fusion protein level itself, but rather acts in a different way. Cell death was induced via apoptosis by the drug *in vitro* and cell growth was inhibited *in vivo* in a xenograft model. The mode of action, how PKC412 blocks the fusion protein activity, has not yet been characterized.

Screening 639 cell lines for 130 different compounds revealed Ewing cell lines to be most sensitive to PARP inhibitor Olaparib treatment [312]. PARP is an enzyme found upregulated in ES, involved in DNA single strand break repair and transcriptional regulation [312-314]. Several experiments pointed out that PARP might also interact with EWS/FLI1 thereby directly regulating its activity [313, 315]. Different studies have reported PARP inhibitors as having significant effects on Ewing cell lines *in vitro*, but unfortunately this is not true for *in vivo* xenograft studies [313]. Indeed PARP inhibitors alone are not sufficient to block tumour growth *in vivo*, but combinations with standards chemotherapeutic agents are needed [316-318]. Since it is not yet clear how PARP is regulated and the sensitivity to Olaparib differs among different cell lines, more studies are required.

From a drug screening performed on the cancer cell line encyclopaedia, Ewing cell lines were identified to be the most sensitive ones to Irinotecan treatment [319]. Irinotecan is a camphotecin analogue that has Topoisomerase I as a target, induces DNA damage and subsequent cell death. Specificity of camphotecin for ES emerged already from another drug screening where EWS/FLI1 gene expression was surprisingly down regulated upon treatment [170].
This class of compounds was isolated from the bark of a Chinese tree named *Camptotheca acuminate* [320] and is currently under study for Ewing sarcoma therapy. Indeed, topoisomerase inhibitors such as Topotecan are already used in combination with normal therapeutics or as a component of the standard regimen [269, 321, 322]. A study of Topotecan treatment in Ewing

sarcoma, Rhabdomyosarcoma and Wilm's tumour revealed a free tumour rate of 86% in xenograft models of paediatric cancers [323] and a response in high-risk patients of 8% when given alone and of 57% when in combination with Cyclophosphamide or Temozolamide [86]. Despites these data, after Topotecan treatment myelosuppression occurs in more than 50% of the patients [324]. Irinotecan instead has a lower rate of side effects and similar or even higher efficacy in Ewing sarcoma alone or in combination with Temozolamide or Trabectedine [325-327]. How camphotecin regulates EWS/FLI1 expression needs still to be uncovered, and the fact that Irinotecan causes only small side effects suggests that its use could be beneficial for Ewing sarcoma treatment.

3.6.3 Targeting CD99

Another protein overexpressed in Ewing sarcoma and also used as diagnostic marker, is the trans membrane glycoprotein CD99. Encoded by the MIC2 gene, it is expressed in normal tissues such as hematopoietic stem cells, testis, prostate, pancreatic cells and gastric mucosa and is expressed also in some malignancies like lymphoblastic lymphoma, embryonal rhabdomyosarcoma, synovial sarcoma, and other soft tissue sarcomas [328-331]. It is known to be involved in cell adhesion [332-334], migration [335, 336], apoptosis [336] and neural differentiation [337]. Engagement of CD99 by a monoclonal antibody induces massive apoptosis in Ewing sarcoma cells by a caspase independent mechanism [330, 338], indeed there are now evidences that cell death might occur by autophagy. This suggests that CD99 is not only a marker but that it has a functional role in the disease and therefore could also serve as target for therapy. Indeed silencing of CD99 affects Ewing cells growth, colony formation capability, reduced motility *in vitro* [337]. More interestingly, blocking CD99 induces tumour delay and regression, the metastatic potential is reduced and cells are more sensitive to chemotherapeutic agents [337, 339, 340]. A combination of Doxorubicin or Vincristine and anti-CD99 treatment resulted in a synergistic and striking lowering of tumour growth of bone/lung metastasis [340].

Clinical trials using CD99 antibody are not attempted yet, even though no strong bone marrow toxicity was noticed. This could have been expected since CD99 is expressed also in hematopoietic cells, pancreas and gonads and it raises some concerns about its use for therapy.

3.6.4 Targeting interactions with cofactors

Another way to block EWS/FLI1 activity could be by inhibiting its interaction with other proteins. Several evidences indeed suggest that EWS/FLI1 is involved in protein-protein interactions and targeting those might lead to new strategies for therapy. Unlike enzymes that can be easily targeted by blocking their ATP binding site, transcription factors require more specific approaches [341]. Study of the interactome of the fusion protein using a phage display library with recombinant EWS/FLI1 identified RNA helicase A (RHA), among others [342], as directly interacting molecule [343]. Disruption of this binding using a derivative of the peptide, reduced cell growth *in vitro* specifically in Ewing sarcoma cells that express the fusion protein EWS/FLI1. No effects were observed in other tumour cell lines from neuroblastoma or rhabdomyosarcoma [344]. Thanks to surface plasmon resonance technology it was possible to identify a small molecule interacting with EWS/FLI1 with similar structure to RHA capable of disrupting the interaction. The (S)-YK-4-279 molecule indeed competes with RHA for the binding to EWS/FLI1 and therefore blocks its activity both *in vitro* and *in vivo* [345, 346]. This is the very first molecule that targets directly the fusion protein and is now under study to improve its half-life and stability in order to progress to clinical trials [347]. A problem might be that the compound also inhibits the binding of endogenous FLI1 and thereby might create side effects in the vascular and hematopoietic system. Encouraging however, current studies have shown that combination of (S)-YK-4-279 with Enzastaurin, an inhibitor of PKC isoforms, found overexpressed in ES, has a synergistic effect in blocking cell proliferation suggesting further combination therapies as an option [347].

3.6.5 Epigenetic modifiers

Since EWS/FLI1 acts as transcription factor and the structure of chromatin is necessary for fusion protein activity, investigators have focused on epigenetic targets.

Marks on histone tails determine whether that gene will be expressed or not and there are three classes of epigenetic modifiers (Fig. 10) [348], namely writers, readers and erasers.

-Writers: to this class belong 100 different enzymes that introduce posttranslational modifications to histone tails such as methylation, phosphorylation or acetylation [349, 350]

-Readers: proteins that recognize the marks

-Erasers: enzyme that reverse the modifications inserted by the writers [351]

Mutations in epigenetic modifiers are rare in ES [352] nevertheless some were found dysregulated and responsible for alteration in gene expression, malignant transformation and might also be involved in drug resistance [353]. From a study mapping the epigenome, it emerged that EWS/FLI1 activated target genes have high levels of open-chromatin-associated marks such as H3K4me3, H3K27ac and H3K56ac and that there are several clusters of target genes according to their epigenetic signature [354].

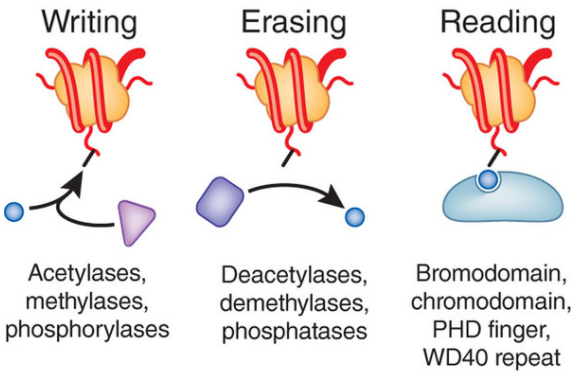

Fig.10 *Epigenetic modifiers*. From [348]

The relevance of epigenetic regulation became more and more interesting in the last few years. Currently there are several drugs that inhibit the catalytic domain of writers or erasers under study in clinical trials and some of them are already FDA approved [355-358]. Also in Ewing sarcoma some of these inhibitors were tested. In 2008 it was demonstrated that the repression exerted on NKX2.2 target genes involved histone deacetylase (HDAC) recruitment, an enzyme belonging to the class of erasers. NKX2.2 recruits HDAC at the promoter level of target genes and therefore the expression is suppressed. HDAC inhibitors like Vorinostat, repress the NKX2.2 signature and inhibit cell growth [144]; such inhibitors were tested in ES xenograft models and tumour growth was reduced using the compound as a single agent [359]. Similar observations were made by others [148, 360] and HDACi were identified also from a small molecule screen based on chromatin accessibility [361].

In the same way, EWS/FLI1 represses LOX expression by interaction with the NuRD complex containing LSD1 and HDAC, and consequently treatment with Vorinostat and/or LSD1 inhibitors induce LOX upregulation [150]. Unfortunately, even if some HDAC inhibitors are already FDA approved, resistance can occur [362]. Another evidence that enlightens the importance of epigenetic modifiers in ES is that the fusion protein regulated EZH2 gene expression which is involved in metastasis and tumour formation [363]. EZH2 is a methyltransferase, an enzyme that is a component of the polycomb-repressor complex 2 (PCR2) and belongs to the class of writers [364]. It functions as transcriptional repressor and is important in many physiological as well as pathological processes [365]. It has also been described to act as oncogene in a variety of cancers [365, 366].

Besides inhibitors for the class of writers and erasers, nowadays more important for cancer treatment is the class of reader inhibitors. Examples are JQ1 and I-BET151, small molecule inhibitors able to disrupt protein-protein interaction between bromodomain (BRD) and acetylated lysine on histone tails, thereby blocking gene expression (Fig. 10A) [367-370].

A variety of 57 bromodomains is present in 41 proteins and is responsible for epigenetic memory as part of transcriptional complexes [371]; proteins containing bromodomains have different functions from acetyltransferase activity to chromatin remodelling and transcriptional activation. A well-known family of bromodomains sharing a common and conserved domain is Bromodomain and extra terminal (BET) family that includes BRD2, BRD3, BRD4 and BRDT [372]. BET proteins promote transcriptional activation, inducing an open chromatin structure and recruiting RNA polymerase [373].

Recently, BRD4 has been described as target for cancer therapy [374-379] due to its direct binding to the promoter of c-MYC which is overexpressed in a variety of cancers [378, 379]. Indeed, it has been shown that upon BRD4 inhibition, the oncogene c-MYC is down regulated and as a consequence also its tumorigenic activity.

The anticancer effect of BET inhibitors has been described in leukaemia [375, 376, 380], osteosarcoma [379], lung cancer [381], prostate cancer [382] and carcinoma [383]. JQ1 was shown to reduce gene expression of genes with strong promoter and super enhancer regulatory region such as c-MYC involved in regulation of oncogenes and rich in bromodomain binding sites [384, 385].

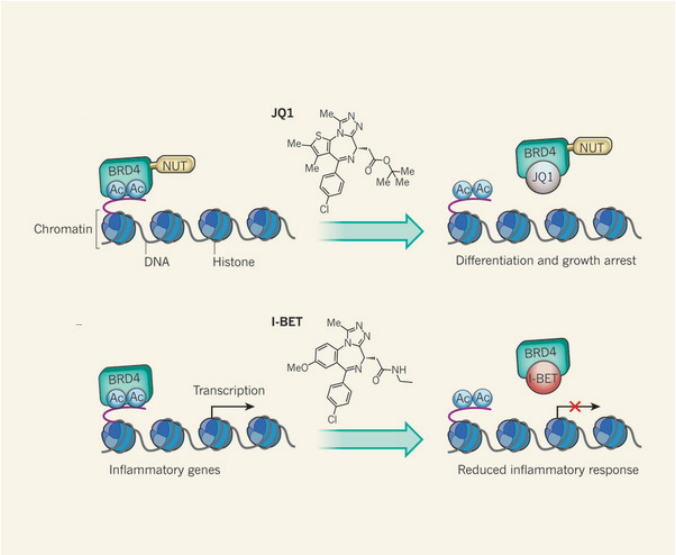

Fig.10 *Reader blockers and PI3K pathway.*
(A) JQ1 and I-BET block the interaction between BDR4 proteins and acetylated hystone's tails. This inhibition leads to a differentiation and growth arrest in the upper panel after treatment of tumor cells. In the lower panel I-BET treatment reduces gene expression of proinflammatory genes [369]

3.6.6 Targeting post-translational modifications

The activity of most transcription factors is regulated by posttranslational modifications and complex formation [386]. Therefore, also EWS/FLI1 is known to be subject of posttranslational modifications such as phosphorylation [387-389], glycosylation [390] acetylation [391] and methylation [392]. It has been shown that methylation alters its cellular localization [392] whereas glycosylation reduces binding to the DNA [390]. Recently, different sites have been described to be phosphorylated such as Threonine 79 in response to DNA damage by JNK and ERK [387] and Serine 266 by PKC kinase family [388]. In a recent publication, it was shown than treatment with Englerin A, a natural molecule isolated from *Phyllanthus engleri*, can inhibit EWS/FLI1 activity by reducing its phosphorylation [389]. Indeed, expression of target genes was reduced as well as the binding of the fusion protein to core promoter regions this reduces cell viability by induction of necroptosis and apoptosis. Targeting posttranslational modifications, by blocking the upstream enzymes, could open a new strategy for therapy to be combined with the ones in use today.

3.7 Cell of origin

Ewing sarcoma is characterized by undifferentiated cells and is generally considered an embryonal tumour, also due to the age of patients. Nevertheless the cell of origin of this tumour has not yet been identified and is still controversially discussed. The different sites of sarcoma appearance according to the age of the patient might suggest different progenitor cell types or a different timing in the progenitor transformation. The initial hypothesis postulated by James Ewing was that the sarcoma was originating from the endothelium. Nowadays, the most accredited possible cell of origin is either mesenchymal stem cell (MSC) [162, 393-398] or neural crest stem cells, since several peripheral neuroectodermal tumours belong to the Ewing sarcoma family of tumours and since ES shows some neural and neuroectodermal marker expression [328]. One would think that one option excludes the other, but recent publications showed that bone marrow derived MSC might originate from neural crest intermediate cells [399-401].

Since the translocation is present only in tumour cells and it is thought to be the driver mutation. To identify the cell of origin of ES, several researchers induced EWS/FLI1 gene expression in different cell types, since the same technique led to the identification of the cell of origin for different sarcoma including alveolar rhabdomyosarcoma [402, 403], synovial sarcoma [404] and osteosarcoma [405].

However, in general there are no adult differentiated cells tolerate EWS/FLI1 expression [232, 406, 407]. Therefore the only permissive cell types were of embryonal origin such as MSC, human embryonal stem cell-derived neural crest stem cells (NCSC), mouse embryonic fibroblast cells (NIH3T3), Human Embryonic Kidney 293 cells (HEK293), Human foetal lung cells (IMR90), C3H mouse embryo cells (C3H10T1/2) [82, 147, 400, 408]. The very first attempts were performed in NIH3T3 murine fibroblast cells [145, 146], where expression of the fusion protein resulted in expression of EWS/FLI1 target genes [246, 409-412]. However, the expression signature overall was not similar to the signature of ES patients [413-417]. Introduction of EWS/FLI1 in human progenitor cells produced similar results, suggesting that fusion protein activity depends on history of the host cell.

Several pieces of evidence support the MSC hypothesis: MSCs colocalize with sites of ES appearance. Second depletion of EWS/FLI1 induces a transcriptional pattern very similar to the one of MSCs [118, 120, 394, 397, 418]. On the other hand, upon treatment with differentiation agents, Ewing cells undergo neural differentiation [419, 420], supporting the NCSC hypothesis.

Expression of EWS/FLI1 in NCSC mimics the ES gene expression signature more than any other transfected cell types [396]. In fact, Ewing cells have some neural marker constantly up regulated. Surprisingly, expression in neuroblastoma cells resulted in silencing of tumour specific markers and upregulation of the ones specific for Ewing, suggesting that EWS/FLI1 is responsible for blocking cell differentiation [421]. This particular observation might suggest that the neural markers expressed in ES simply result from EWS/FLI1 expression and not because of the cellular background. The same occurs when EWS/FLI1 is overexpressed in rhabdomyosarcoma cells, where the morphology of cells changes into small round cells and muscle differentiation markers are silenced and the ones of ES upregulated [422]. Indeed it looks like expression of the fusion protein has the capability to block cell differentiation [120]. Also, overexpression of EWS/FLI1 in MSC has been associated with upregulation of those genes involved in pluripotency such as SOX2, NANOG and OCT4 [394] and upregulation of the polycomb repressor EZH2 [363].

The presence of the fusion protein in human MSCs did not result in any oncogenic transformation, whereas when transfected in murine MSCs it did [162]. Recently, it was published that overexpression of EWS/FLI1 in murine osteochondrogenic progenitors engrafted into mice led to formation of a tumour with ES signature, suggesting this cell type as potential cell of origin [423]. Unfortunately, comparison of murine ES with the human one did not match to 100%, indicating that the road is still long. One year later, the same group tried to generate a mouse model of ES using the Cre Lox system to recreate the original translocation. However, none of the mice developed tumours but cardiomyopathy was induced [424]. A similar study was conducted inducing EWS/FLI1 expression in bone marrow stromal cells, but the result was an osteosarcoma [425]. Further expression of the fusion protein in murine MSC led to development of leukemia and not Ewing tumour [426]. Another approach was used by American investigators: they induced expression of EWS/FLI1 in embryoid bodies generating cells with a strong ES signature, but again when engrafted into mice, no tumour was formed [427].

For this reason, the search for a host cell where EWS/FLI1 expression can drive tumorigenesis *in vivo* is still on going. Despite all these observations that give first hints to the cell of origin, the presence of the fusion protein would need to produce a sarcoma when engrafted into mice. Indeed no genetically engineered mouse model has been developed so far to study the development and maintenance of the tumour.

Knowing the cell of origin would be a huge step forward to understand the pathology of the disease and pre-clinically investigate novel therapeutic strategies.

4. Aim of the thesis

Ewing sarcoma is an aggressive osteolytic tumor that affects children and young adults. It is characterized by a balanced translocation between chromosomes 11 and 22, generating a dysregulated transcription factor EWS/FLI1 where the transactivation domain of EWSR1 is fused to the DNA binding domain of FLI1.

EWS/FLI1 is expressed only in cancer cells and is responsible for tumorigenesis and tumour maintenance through regulation of its target genes. Therefore, it represents an ideal target for therapy. Unfortunately being a transcription factor it is considered undruggable since it lacks enzymatic activities, and consequently there are no direct ways to inhibit its function, so novel indirect therapeutic approaches are needed.

For these reasons we aimed in this thesis at investigating different strategies to target EWS/FLI1 in Ewing sarcoma.

Aim n.1: TARGETING EWS/FLI1 EXPRESSION THROUGH MODULATION OF SIGNALLING PATHWAYS

From a drug screening with 153 targeted compound we identified PI3K inhibitors as modulators of EWS/FLI1 activity. From these, BEZ235 was selected for further investigation. Treatment with BEZ235 led to downregulation of EWS/FLI1 gene expression itself. Therefore, we aimed at identifying and characterizing a possible transcription factor regulated by the PI3K pathway which might bind to the promoter region of EWSR1. This would lead to the identification of a new target for therapy.

Aim n.2: TARGETING EWS/FLI1 EXPRESSION BY EPIGENETIC MECHANISMS

Since EWS/FLI1 is an oncogene and since BRD4 inhibitors emerge as modulators of oncogene expression, we aimed at testing JQ1, a BRD4 inhibitor, in Ewing cells.

AIM n.3: TARGETING EWS/FLI1 ACTIVITY

Posttranslational modifications are known to affect protein activity, likely also in EWS/FLI1.

We aimed therefore at investigating how inhibition of EWS/FLI1 phosphorylation, described to be necessary for the complete function of the fusion protein, was affecting the fusion protein activity.

Knowledge of molecular mechanisms regulating EWS/FLI1 activity can lead to development of new strategies for a targeted therapy specific for Ewing sarcoma.

5. Publications

5. 1 PI3K/AKT signaling modulates transcriptional expression of EWS/FLI1 through specificity protein 1

Manuscript published in Oncotarget on August 22, 2015

Contribution: AB performed screening together with FR, in vitro assays and promoter analysis. I further performed drug treatments, silencing of putative transcription factor candidates, immunofluorescence staining, EMSA and ChIP assays, Cell cycle analysis, Caspase 3/7 assay and Crystal violet staining (Fig. 1B,C/ Fig. 2D/ Fig. 4/ Fig.5/ Fig.6/ Fig. 7/ Suppl. Fig. 1/ Suppl. Fig. 2/ Suppl. Fig. 3B-E/ Suppl. Fig. 4/ Suppl. Fig. 5/ Suppl. Fig. 6/ Suppl. Fig. 7/ Suppl. Fig. 8/ Suppl. Fig. 9/ Suppl. Fig. 10).

AB and I contributed equally in writing this manuscript.

PI3K/AKT signaling modulates transcriptional expression of EWS/FLI1 through specificity protein 1

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ABSTRACT

Ewing sarcoma (ES) is the second most frequent bone cancer in childhood and is characterized by the presence of the balanced translocation t(11;22)(q24;q12) in more than 85% of cases, generating a dysregulated transcription factor EWS/FLI1. This fusion protein is an essential oncogenic component of ES development which is necessary for tumor cell maintenance and represents an attractive therapeutic target. To search for modulators of EWS/FLI1 activity we screened a library of 153 targeted compounds and identified inhibitors of the PI3K pathway to directly modulate EWS/FLI1 transcription. Surprisingly, treatment of four different ES cell lines with BEZ235 resulted in down regulation of EWS/FLI1 mRNA and protein by ~50% with subsequent modulation of target gene expression. Analysis of the EWS/FLI1 promoter region (−2239/+67) using various deletion constructs identified two 14bp minimal elements as being important for EWS/FLI1 transcription. We identified SP1 as modulator of EWS/FLI1 gene expression and demonstrated direct binding to one of these regions in the EWS/FLI1 promoter by EMSA and ChIP experiments. These results provide the first insights on the transcriptional regulation of EWS/FLI1, an area that has not been investigated so far, and offer an additional molecular explanation for the known sensitivity of ES cell lines to PI3K inhibition.

INTRODUCTION

Ewing sarcoma (ES) is the second most frequent bone cancer in childhood. Clinically, ES appears as very aggressive osteolytic tumor with early tendency for development of metastasis [1]. It belongs to the group of small-round-blue-cell tumors and is comprised of largely undifferentiated cells. The unique feature of this tumor is the presence of the balanced t(11;22)(q24;q12) translocation in more than 85% of cases [2]. This gene rearrangement results in expression of a chimaeric fusion protein where the RNA binding domain of EWS is exchanged by the DNA binding domain of the ETS transcription factor FLI1, thus generating an aberrant transcription factor EWS/FLI1 [3–6]. More than 18 less represented alternative translocations involving EWS and other ETS protein family members have been described since [7–12].

Extensive evidence supports the notion that EWS/FLI1 is an essential oncogenic component of ES development. Its oncogenic activity is thought to be mediated through inappropriate regulation of target genes that are crucial for the fully malignant phenotype [5, 6, 13–17]. So far it is not known which of this target gene(s) act as crucial oncogenic driver(s). Hence, the prevalent hypothesis states that EWS/FLI1 is the major genetic mutation that is necessary for development and maintenance [18–24] of the tumor although it might not be sufficient. Since EWS/FLI1 expression is restricted to tumor cells, it represents an ideal therapeutic target. However, it acts as transcription factor, which in most cases are considered undruggable because of lack of enzymatic activity and their direct pharmacological inhibition is still challenging. Indeed, EWS/FLI1 behaves as intrinsically disordered protein and so far cannot be directly targeted by small molecules in a classical sense.

Therapy of ES today lacks specificity, is ineffective against metastasis and bears the potential of serious side effects. In the last few decades there has been considerable progress in both diagnosis as well as treatment of localized disease. However, only 15% of patients with metastatic disease survive and therefore this patient group needs specific attention. To advance future therapies, one of the available options lies in a better understanding of the biology of the fusion protein. Considering the difficulties in finding direct small molecule inhibitors for transcription factors, our aim is to study and characterize the cellular processes affecting or being affected by the fusion protein and thus providing indirect targeting possibilities. To identify molecular pathway(s) that might contribute to the transcriptional activity and oncogenic properties of EWS/FLI1, we therefore adopted a screening approach previously described [25], and screened a small molecule library that includes a broad range of protein kinase inhibitors covering all major signaling pathways. This approach led to the identification of SP1 as a direct regulator of EWS/FLI1 transcription through activation via the IGF/PI3K/AKT pathway, which is known to play a role in Ewing sarcoma [26–32] and whose blockage affects cell growth and survival [33–37]. Hence, we identify a critical regulatory mechanism upstream of EWS/FLI1.

RESULTS

Screening a library of small molecule inhibitors identifies PI3K pathway as modulator of EWS/FLI1 expression

To identify molecular pathways that may contribute to transcriptional activity and oncogenic properties of EWS/FLI1, we used the previously established and validated strategy [25] to screen a library of small molecule inhibitors covering a wide variety of molecular pathways. The collection of 153 inhibitors (Supplementary Table S1) was screened for EWS/FLI1 target gene modulation as primary read out in A673 ES cells at a final concentration of 500 nM. EWS/FLI1 transcriptional activity was monitored by expression of the known target genes pleckstrin homology-like domain, family A, member 1-PHLDA1 [25], Nuclear Receptor Subfamily 0 Group B Member 1-NROB1 [20], NK2 homeobox 2-NKX2.2 [21] and for EWS/FLI1 itself. Expression of PHLDA1 is repressed by EWS/FLI1, in contrast to NROB1 and NKX2.2 which are activated. General cytotoxicity of the compounds was determined by WST-1 assay, a colorimetric assay based on the cleavage of a tetrazolium salt, to form formazan in viable cells. The final hit-list was based on significant ($p < 0.05$, unpaired two-tailed t-test) modulation of at least two out of three target genes compared to untreated controls in A673 cells. The top 16 inhibitory compounds obtained from the screen are shown in Table 1 and included inhibitors targeting several signaling pathways, both known and unknown to play a role in sarcomas. The most prominent among them is the phosphoinositide-3-kinase (PI3K) pathway, which was affected by three different compounds. Inhibition of this pathway provoked a significant modulation of EWS/FLI1 target genes and a strong inhibition of cell proliferation in A673. Hence, these experiments identified PI3K signaling to modulate expression of EWS/FLI1 target genes.

Among the PI3K inhibitors tested was BEZ235, which is a dual inhibitor of PI3K and the downstream mammalian target of Rapamycin (mTOR) that induced the most significant modulation of all three EWS/FLI1 target genes. Hence, we focused on this compound to further characterize modulation of EWS/FLI1 activity by the PI3K-mTOR pathway. Interestingly, upon treatment of four ES cell lines with 500 nM BEZ235 we observed a decrease of more than 50% of EWS/FLI1 mRNA levels itself (Figure 1A) that also resulted in a reduction of EWS/FLI1 protein levels (Figure 1B, 1C and Supplementary Figure S1) As expected, decrease of EWS/FLI1 mRNA led to modulation of target gene expression as well (NKX2.2, NROB1 and PHLDA1). Additional target genes such as insulin-like growth factor binding protein 3- IGFBP3 [19] and Lysyl Oxidase -LOX [38], repressed by EWS/FLI1, and six transmembrane epithelial antigen of the prostate 1-STEAP1 [39] and protein kinase C Beta -PRKCB [40], activated by EWS/FLI1, were found to be modulated as well (Supplementary Figure S2A–S2D).

Therefore, this data suggests that PI3K signaling is involved in transcriptional regulation of EWS/FLI1 expression.

BEZ235 treatment induces cell cycle arrest

As described above, treatment with 500 nM BEZ235 for 24 hrs resulted in a decrease of EWS/FLI1 protein levels (Figure 1B and 1C) and as a consequence in PHLDA1 upregulation, which in turn led to a dose dependent reduction of viable cells compared to non-treated controls (Supplementary Figure S3A). To verify that the drug affected cell proliferation we stained the cells with crystal violet after drug treatment with 500 nM BEZ235 for 24 and 48 hrs. We observed a reduction of cell numbers by 40% and 70% compared to the DMSO control in A673 and 48% and 77% in SKNMC cells. Nevertheless, reduction in cell numbers was much more pronounced when cells were treated with Staurosporin or Nocodazole (Supplementary Figure S3B–S3E). Hence, BEZ235 seems to affect cell proliferation without decreasing viability. To investigate whether the compound induces cell death, we investigated PARP cleavage by Western blot. As shown in Figure 1B treatment with 500 nM BEZ235 resulted in minor PARP cleavage only. Subsequently, we investigated Casp3 and 7 activity both with the Casp3/7 Glo assay and at protein levels (Supplementary Figure S4A and S4B and data not

Table 1: Screening of a small library of targeted compounds identifies PI3K pathway inhibitors as modulators of EWS/FLI1

			EWS/FLI1 target gene modulation in A673 cells*,**					
Compound[a]	Target	Company	EWS/FLI1[b]	PHLDA1	NROB1	NKX2.2	Nr. of sign. Target gene response**	Proliferation[c]
NVP-BEZ235	PI3K/mTOR inhibitor	Axon 1281	89	**232**	**65**	**40**	**3**	58
PIK 75	PI3K/p110 alpha inhibitor	Axon 1334	**8**	115	9	**5**	**2**	54
NPV-BAG956	PI3K/PDK1 inhibitor	Axon 1282	111	**176**	83	**68**	**2**	68
DBZ	Gamma Secretase inhibitor	Axon 1488	116	**183**	**85**	**68**	**3**	113
BZ	Gamma Secretase inhibitor	Axon 1487	102	**181**	88	**77**	**2**	115
Vorinostat	HDAC inhibitor	Cayman	**47**	**185**	**61**	**68**	**3**	124
Bosutinib (SKI 606)	BCR-ABL/SRC inhibitor	Axon 1407	92	**221**	**84**	**56**	**3**	121
Tacrolimus	Calcineurin inhibitor	Axxonra	62	**126**	**71**	**67**	**3**	106
YM155	Survivin inhibitor	Selleck 1130	**25**	120	**28**	**46**	**2**	22
LY 2157299	TGF beta inhibitor	Axon 1491	73	153	**76**	**68**	**2**	108
Velcade	Proteosome inhibitor	Cilag	77	119	**17**	**18**	**2**	45
ICG-001	CBP/Beta-Catenin inhibitor	Selleck 2662	61	109	**72**	**69**	**2**	100
GDC-0449	Hedgehog Pathway inhibitor	Selleck 1082	97	147	**86**	**63**	**2**	114
Tandutinib	FLT3 inhibitor	Axon 1415	61	**137**	105	**66**	**2**	103
TG101348	JAK2 inhibitor	Symansis	**59**	**128**	97	**77**	**2**	100
NU 1025	PARP inhibitor	Axon 1370	90	**130**	87	**81**	**2**	120

[a]Treatment: 500 nM of compound for 24 hrs

[b]Relative mRNA expression levels of EWS/FLI1 and its target gene in percentage compared to DMSO. Significant values are written in bold ($p < 0.05$, unpaired two-tailed student *t*-test).

[c]Cell proliferation measurement using WST-1 assay was performed in parallel. Values are shown in percentage of untreated control (=100%) and represent mean of 2–4 independent experiments performed in duplicate.

*In % of control (=100%)

**$p < 0.05$, unpaired two-tailed *t*-test, significant values written in bold.

shown). We observed no increase in activity of Casp3/7 after BEZ235 treatment, in contrast to treatment with Staurosporin and Nocodazole used as positive controls (increase by 5–6 fold). Hence, BEZ235 treatment did not induce apoptosis as measured by caspase activation and PARP cleavage. Subsequent cell cycle analysis after treatment with 500 nM BEZ235 for 24 and 48 hrs, both in A673 and SKNMC cells, revealed an increase in the cellular fraction in G1 phase. Indeed, the G1 population raised by 20% in A673 and 30% in SKNMC cells after drug treatment compared to DMSO control (Supplementary Figure S5A and S5B). Taken together,

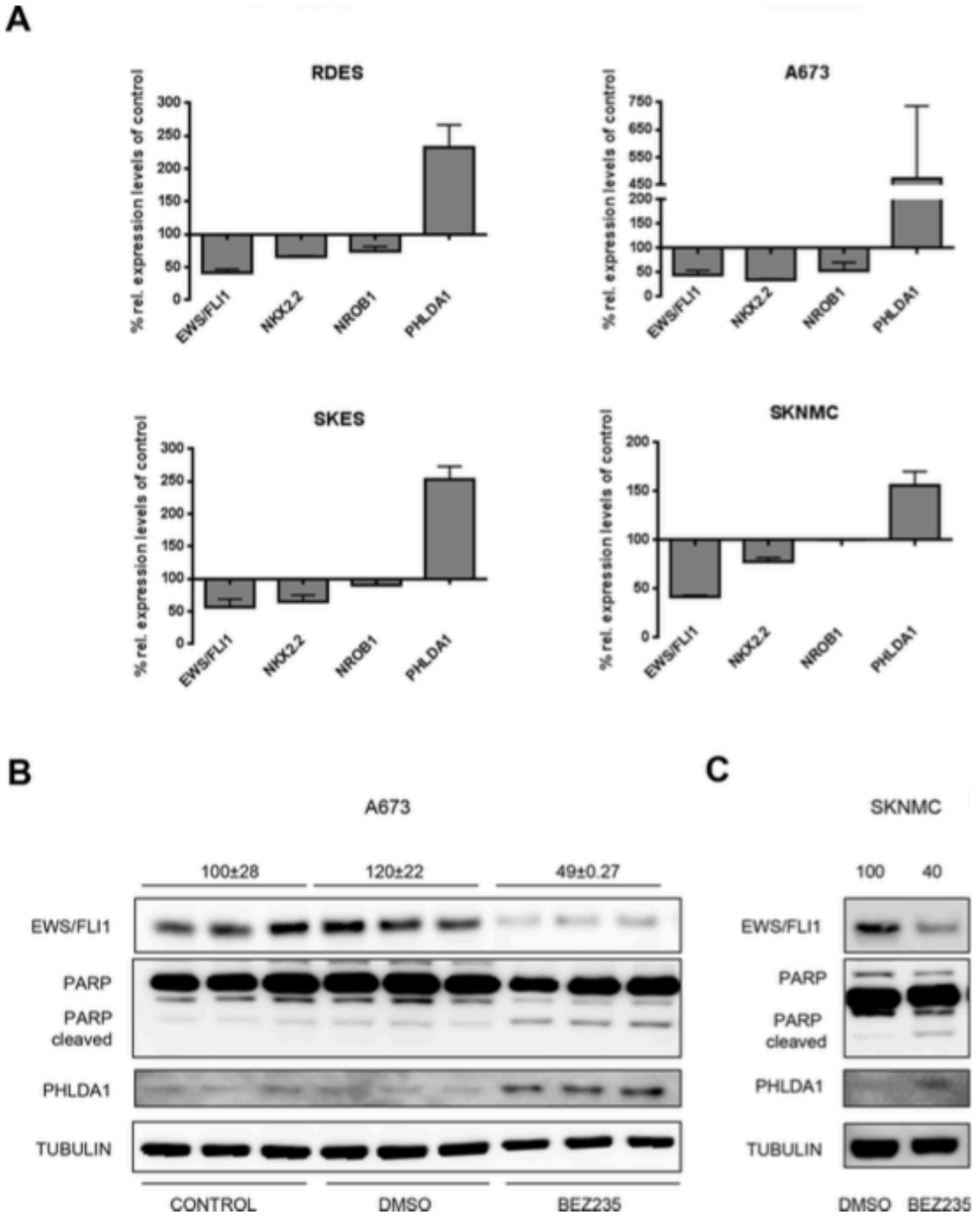

Figure 1: BEZ235 affects EWS/FLI1 levels. A. Relative expression of EWS/FLI1 and its target genes measured by qRT-PCR after 24 hrs treatment with 500 nM BEZ235. Mean and standard deviation of 3 independent experiments. **B.** Protein level measured by western blot of EWS/FLI1, PHLDA1, PARP and TUBULIN as loading control in biological triplicates. Cells were treated for 24 h with 500 nM BEZ235 in A673 (B) and SKNMC cells **C.** (Numbers on top of the blot represent intensity of EWS/FLI1 bands measured by densitometry, indicated as mean compared to control and normalized to tubulin).

we conclude that BEZ235 treatment induces a cell cycle arrest, similar to what has been reported earlier [24]. Since the effect of BEZ235 on cell cycle progression could be due to inhibition of PI3K pathway or to EWS/FLI1 reduction, we also investigated the role of EWS/FLI1 in cell cycle progression. We depleted EWS/FLI1 both in A673 and SKNMC using 10 nM of siRNA for 48 hrs and analyzed the cell cycle distribution. Our results showed that depletion of EWS/FLI1 does not induce cell cycle arrest (Supplementary Figure S6A and S6B), but rather provokes a subG1 peak in SKNMC cells. This was also validated at protein level where EWS/FLI1 depletion provoked Casp7 activation and PARP cleavage in SKNMC (Supplementary Figure S6D), but not in A673 cells (Supplementary Figure S6C). In addition, crystal violet staining showed a reduction in cell numbers by 40% in SKNMC cells (Supplementary Figure S6F), but no effect on A673 cells (Supplementary Figure S6E). Taken together these data show that BEZ235 affects cell numbers mainly by inducing cell cycle arrest in ES cells.

PI3K protein depletion reduces EWS/FLI1 expression

To exclude off-target effects of the small molecule inhibitor BEZ235, we performed genetic loss-of-function experiments using siRNA targeting the catalytic domains α, δ and γ of class I PI3Ks in A673 and SKNMC cells. Silencing for 48 hrs resulted in down regulation of PI3K α, δ and γ mRNA by 75% compared to scrambled control as measured by quantitative RT-PCR (Figure 2A). In silenced cells PHLDA1 was up regulated by 10 fold, whereas target genes NROB1, NKX2.2 and Caveolin1 [41] were repressed by 70%, 55% and 45%, respectively (Figure 2B). Notably, expression of EWS/FLI1 itself was inhibited by 65%, whereas only a non-significant alteration of wt FLI1, used as negative control, was observed. These results were confirmed at the protein level since 48 hrs after treatment with PI3K specific siRNAs, EWS/FLI1 protein expression decreased while PHLDA1 protein level increased. As expected, silencing of PI3K decreased phosphorylation of its downstream effectors AKT, mTOR and S6 ribosomal protein as shown by phospho-specific antibodies (Figure 2C). The same result was confirmed in SKNMC cells where depletion of PI3Kαγδ led to a decrease of EWS/FLI1 protein levels, whereas the depletion of each single component did not affect the fusion protein (Supplementary Figure S7A). In addition, we performed immunofluorescence analysis to confirm the reduction of EWS/FLI1 after silencing of the PI3K components on the single cell level. As shown in Figure 2D (lower panel), after silencing of the three subunits, EWS/FLI1 is barely detectable anymore. Altogether, these results confirm regulation of EWS/FLI1 transcription by the PI3K pathway also at the genetic level. To validate that the effect on EWS/FLI1 target genes by the compound is due to the presence of EWS/FLI1, we performed the same assays in prostate cancer cells lacking the fusion protein. As shown in Supplementary Figure S7B, target genes of EWS/FLI1, while well expressed at endogenous levels in this cell type, are not affected by PI3K inhibition.

PI3K signaling controls EWS/FLI1 transcription at its promoter

Since levels of wt FLI1 did not change upon inhibition of the PI3K pathway while at the same time EWS/FLI1 mRNA expression was reduced, we hypothesized that this control of EWS/FLI1 transcription occurs within the EWS promoter. To test this notion, we conducted reporter assays with a plasmid containing the 2.3 kb promoter of EWS in front of the luciferase gene. A673 ES cells were transfected with this construct and treated with increasing concentrations of BEZ235 for 24 hrs (Figure 3A). Interestingly, we observed a dose dependent decrease in luciferase activity which was not the case for a constitutive promoter thereby excluding effects of the compound on stability of luciferase itself. Already 50 nM of BEZ235 was able to reduce luciferase activity by 50% without affecting cell viability implying that indeed this promoter region contains a regulatory element responsive to PI3K signaling (Figure 3A). To narrow down the region of interest we designed several deletion constructs and performed reporter assays as before. Even the smallest construct of -275bp still responded to BEZ235 treatment. Hence, a regulatory element must be contained within this promoter element (Figure 3B).

To pinpoint further this potential regulatory element within the −275/+67 region of the promoter, we designed an additional series of deletion constructs in which 12–14bp were deleted in the context of the full size promoter (Figure 3C). If the responsible regulatory element is excised from the promoter we expect to lose any difference in luciferase activity upon drug treatment. Indeed, two constructs out of 24 did not respond to drug anymore, namely deletion 2 and deletion 23 (Figure 3D). Interestingly, both deletions also lost basal activity by more than 50% (Figure 3C). Therefore, our results suggest that inhibition of PI3K pathway affects gene expression of EWS/FLI1 mainly via two regions of the EWS promoter.

SP1 is involved in transcriptional regulation of EWS/FLI1

Our results point to an unknown transcription factor that binds to a specific region in the EWS promoter and whose activity can be stimulated by the PI3K pathway. Therefore, we used the sequence covered by Del23 to identify transcription factor candidates *in silico*. Using the programs Alibaba 2.2 and P-Match based on consensus sequences and Genome Browser based on ChIP datasets, we identified four potential candidates, C-Rel, YY1,

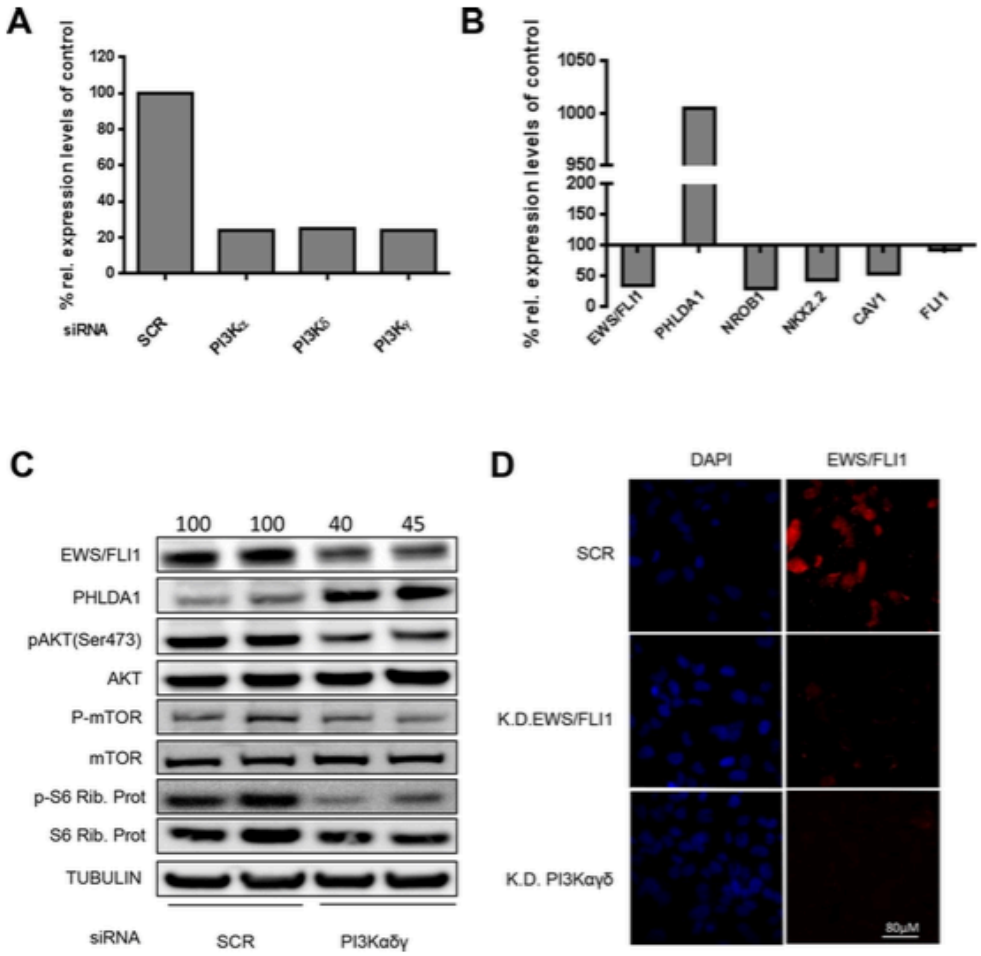

Figure 2: Modulation of EWS/FLI1 and target genes after PI3K pathway silencing. A. PI3K α, γ, and δ mRNA expression levels were measured in A673 cells after silencing for 48 hrs compared to scrambled control by qRT-PCR. **B.** EWS/FLI1 and its target genes mRNA expression upon silencing of PI3K α, γ, and δ in A673 cells for 48 hrs. **C.** Expression levels of EWS/FLI1, PHLDA1 and PI3K downstream effectors after silencing in A673 cells for 48 hrs in biological duplicate (numbers on the top of the blot represent intensity of EWS/FLI1 bands measured by densitometry). **D.** Immunofluorescence assessment of FLI1 after silencing for 48 hrs of PI3Kαγδ; scrambled as control and FLI1 knock down as positive control. All the assays have been performed 3 times, shown are representative experiments.

NFKB and SP1, all known downstream targets of the PI3K pathway (Figure 4A). To determine which of these transcription factors might be involved in EWS/FLI1 gene expression, we performed siRNA depletion of each of the candidates and measured EWS/FLI1 gene expression together with its target gene NROB1 by qRT-PCR (Figure 4B) Knockdown of SP1 led to a reduction of EWS/FLI1 levels by 50% compared to control also at protein level (Figure 4C and Supplementary Figure S8A, S8B), whereas the other candidates did not affect EWS/FLI1 neither at the level of gene expression nor at protein level (Figure 4C and Supplementary Figure S9A–S9D). Also in this case, immunofluorescence analysis after SP1 depletion revealed barely detectable levels of EWS/FLI1, further strengthening the previous observation (Figure 4E and Supplementary Figure S10A and S10B). The same assays have been performed also in prostate cancer cells where SP1 depletion did not affect the levels of the target genes (Supplementary Figure S8C). To further validate our observations, we combined the knockdown of SP1

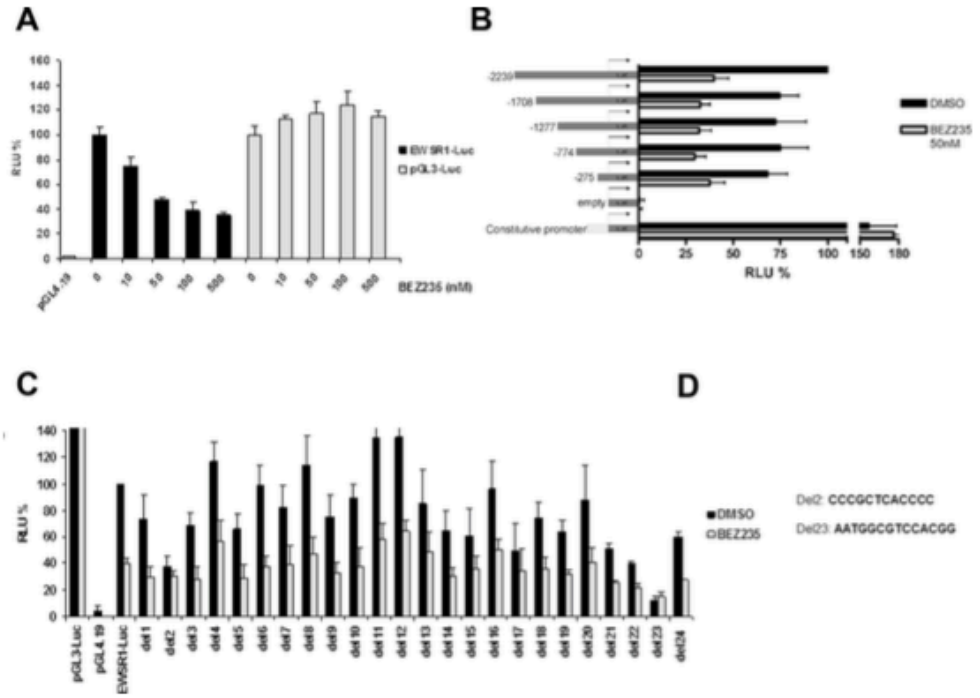

Figure 3: EWS/FLI1 promoter analysis by luciferase assay. A. Relative luciferase activity in A673 cells transfected with different constructs (pGL4.19-empty vector control, pGL3- constitutive promoter control, EWSR1-Luc- 2.3kb promoter vector) upon treatment for 24 hrs with different concentration of BEZ235. **B.** Relative luciferase activity in A673 cells transfected with different deletion constructs of EWS/FLI1 promoter. Cells were treated with either DMSO or 50 nM BEZ235 **C.** Luciferase assay performed as above, with a series of deletion constructs of the 2.3kb EWS/FLI1 promoter **D.** Sequences of the two minimal binding elements that are absent from the deletion constructs Del2 and Del23 Mean and standard deviation of 3 independent experiments.

with 500 nM BEZ235 treatment for 24 hrs and observed an additive reduction of EWS/FLI1 levels (Figure 5A and 5B) both at gene expression and at protein level. Taken together we conclude that PI3K pathway regulates gene expression of EWS/FLI1 through SP1 activity.

To better understand the relation between the PI3K pathway and SP1 in ES cells, we investigated whether its activity is modulated by PI3K signaling. Since knock down of PI3K αγδ subunits induces a similar decrease of EWS/FLI1 mRNA and protein as depletion of SP1 (Figure 4D and 4F), we hypothesized that PI3K could directly affect SP1 levels. Since SP1 is a transcription factor and it is mainly located in the nucleus, we investigated SP1 protein levels after treatment for 24 and 48 hrs with either BEZ235 or Rapamycin, an inhibitor of the mTORC1 complex, in the nuclear fraction. Indeed, we observed a clear decrease of SP1 protein (Figure 6A and 6B). This was confirmed by immunofluorescence stainings showing a clear decrease of SP1 levels after 500 nM BEZ235 treatment also in a non-Ewing cell line such as human foreskin fibroblasts-HFF (Figure 6C). These results suggest that inhibition of PI3K pathway reduces SP1 activity, most likely via phosphorylation dependent mechanisms.

SP1 directly binds to the Del23 region

To demonstrate direct binding of SP1 to the Del23 region of the EWS/FLI1 promoter, we performed electrophoretic mobility shift assays using biotinylated double strand oligonucleotides covering the DNA sequence of Del23 (Figure 7A). Addition of nuclear extract produced a shift that could be competed by addition of an excess of unlabeled Del23 oligonucleotide (Figure 7B, lanes 2, 3), indicating that the Del23 region is indeed bound by protein. This shift could also be competed with an SP1 specific oligonucleotide (lane 4) as well as with a specific antibody against SP1 (lane 5), but not by addition of a control antibody (actin, lane 6). Specificity of the assay was further validated with a mutant Del23

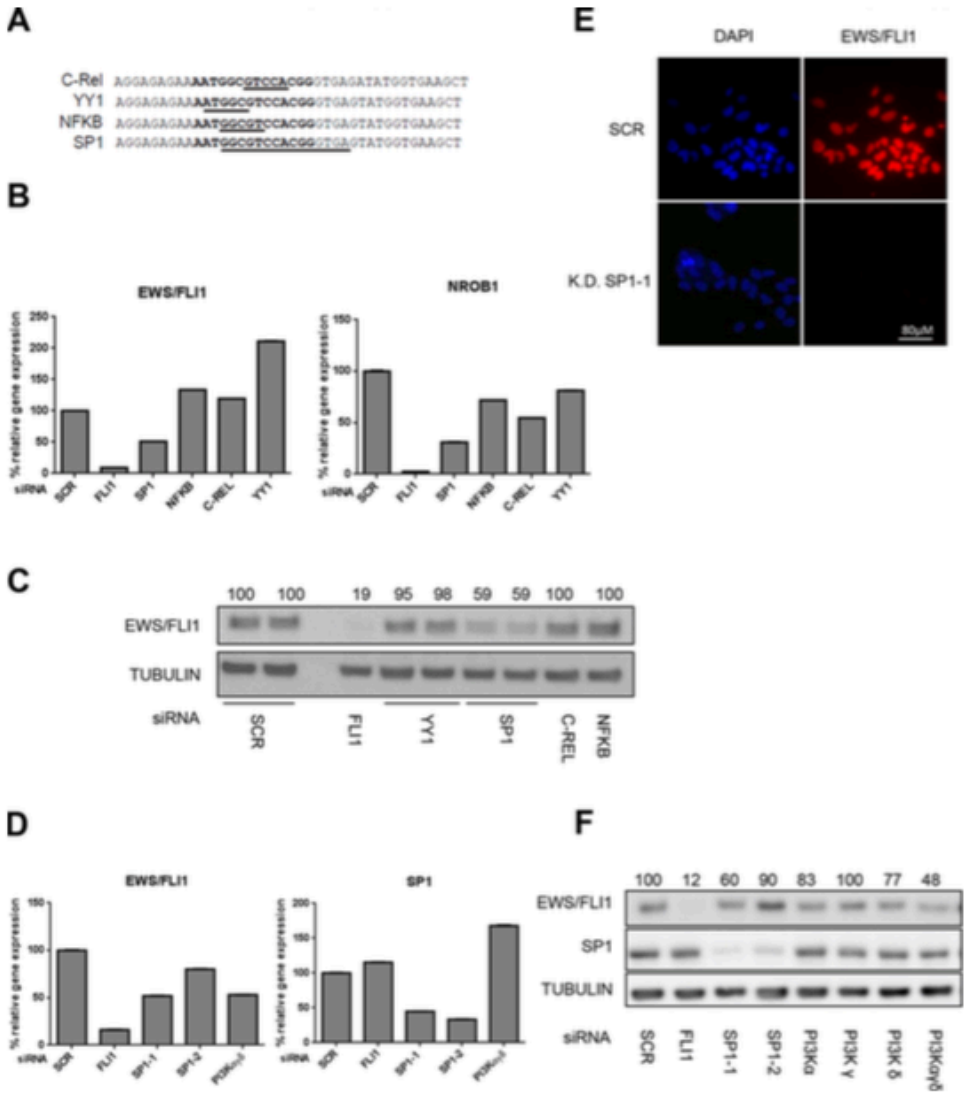

Figure 4: SP1 knock down affects EWS/FLI1 levels. A. List of candidates which may bind the Del23 region of the promoter of EWS/FLI1 identified by Alibaba 2.2, Genome Browser and P-Match programs. **B.** siRNA mediated knock down for 48 hrs of candidate transcription factors to determine mRNA expression of EWS/FLI1 and target genes (via qRT-PCR). **C.** EWS/FLI1 protein level after silencing for 48 hrs of the candidate transcription factors by siRNA. **D.** siRNA mediated knockdown of PI3K components in order to determine mRNA expression of EWS/FLI1, and SP1 (via qRT-PCR) after 48 hrs. **E.** Immunofluorescence assessment of FLI1 after silencing of SP1 for 48 hrs. **F.** EWS/FLI1 protein level measured by western blot after 48 hrs reverse silencing of PI3K single subunits and combinations. Shown are representative experiments ($n = 3$).

oligonucleotide that generated a faint but unspecific shift (lane 8, 9) and with an SP1 specific oligonucleotide that could be displaced with the SP1 specific antibody (lane 13) similar to Del23. Since also Del2 has been implemented in EWS/FLI1 gene expression by the reporter assays and since also this region is GC rich, we tested Del2 in gel shift experiments as well. Del2 oligonucleotide produced a shift that could be competed with unlabeled oligo but not

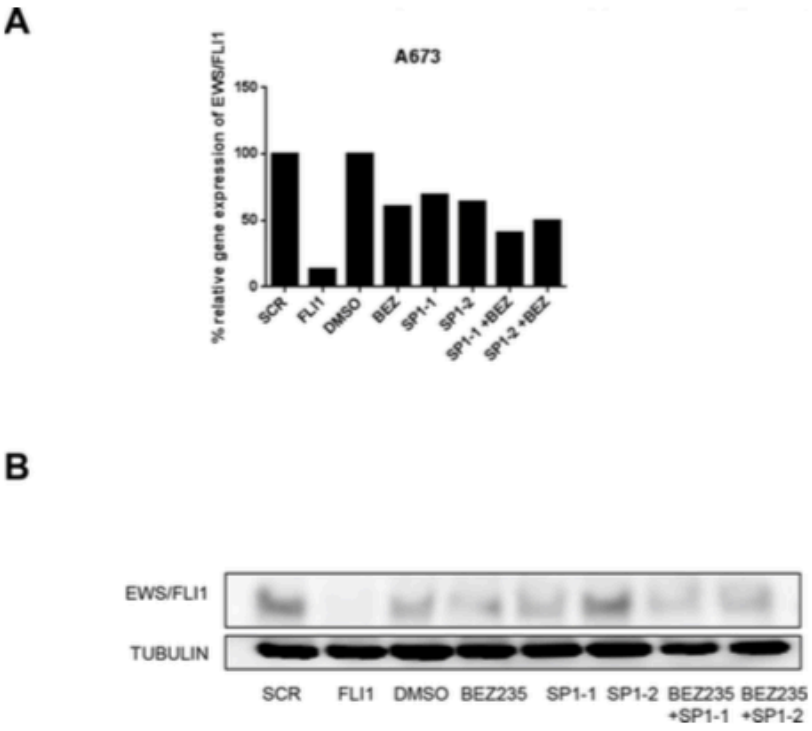

Figure 5: SP1 knock down in combination with BEZ235 treatment affects EWS/FLI1 levels. EWS/FLI1 gene expression l **A.** and protein level **B.** after silencing of SP1–1 and SP1–2 by siRNA for 48 hrs or BEZ235 treatment for 24 hrs or the combination in A673 cells. Shown is a representative experiment ($n = 3$).

by addition of the SP1 antibody (lane 15, 17). Hence, these experiments suggest that the Del23 region of the EWS/FLI1 promoter is bound specifically by SP1 that does not bind to the Del2 sequence.

To verify this notion, we performed chromatin immunoprecipitation assays in two different Ewing cell lines (A673 and SKNMC) (Figure 7C and 7D). Using oligonucleotides spanning the Del23 region of the EWS promoter, a fragment could be enriched by immunoprecipitation with the SP1 antibody as well as with the control H3 antibody, but not with the unspecific IgG control. All together, these results indicate that SP1 indeed binds to the Del23 sequence in the promoter of EWS to modulate its transcriptional expression.

DISCUSSION

Despite increasing efforts there are still no targeted agents implemented in routine therapy of Ewing sarcoma. Recently, several novel targeted approaches have been initiated and underwent clinical trials with limited success [42–45]. However, most of these efforts involved targeting of several enzymes downstream of the EWS/FLI1 fusion protein, such as IGF1R, and did not attempt to modulate the activity of this likely most crucial factor for ES oncogenesis itself.

Here, we conducted a screen of a library of small molecule targeted inhibitors affecting a broad range of different signaling pathways in order to define potential novel nodes directed at the fusion protein. For this, we employed the previously described and well established screening approach that uses expression of three EWS/FLI1 target genes, both repressed as well as activated, as surrogate markers of EWS/FLI1 activity [25]. As the most prominent pathway that was able to modulate EWS/FLI1 target gene expression the P3IK pathway emerged. This came as no surprise, since the importance of the IGFR1-PI3K-AKT axis has been demonstrated already in numerous studies [29, 30, 46–48], and triggered several clinical trials. However, using BEZ235, a dual PI3K/ mTOR inhibitor, as the most potent compound in our hit list we observed a strong decrease in EWS/FLI1 activity and surprisingly this occurs as consequence of a reduction at protein and RNA level of the fusion protein. In addition, since there was a

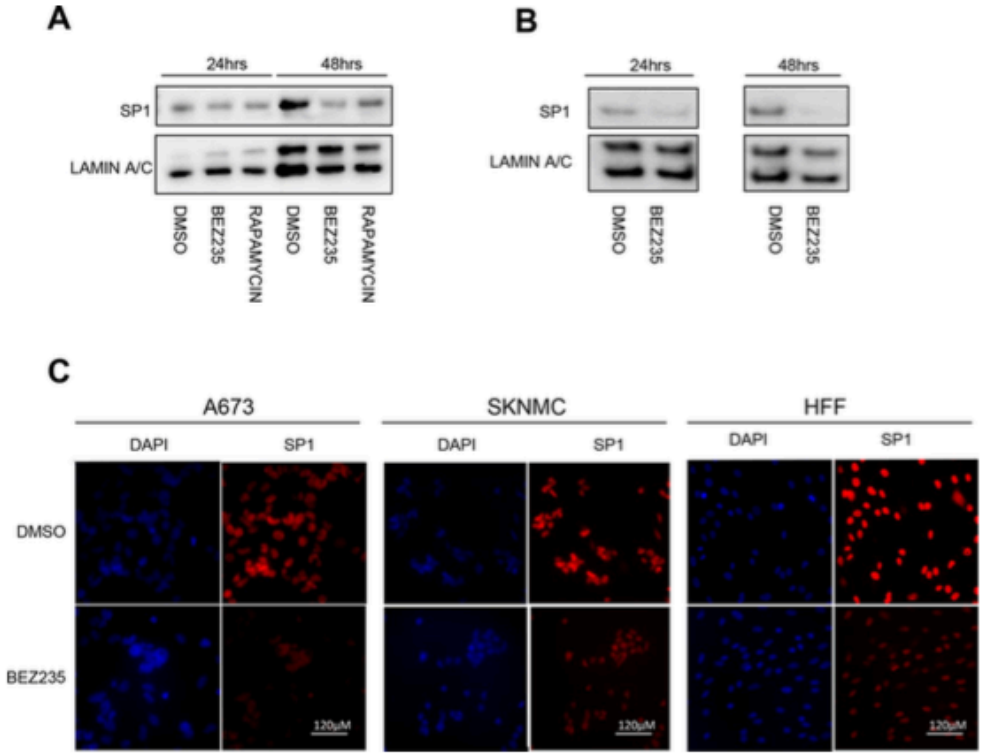

Figure 6: BEZ235 treatment affects SP1 levels. Nuclear extracts of A673 and SKNMC cells were analyzed by western blot after BEZ235 and Rapamycin treatment for 24 and 48 hrs compared to DMSO control in A673 **A.** and in SKNMC using an SP1 specific antibody. **B.** Immunofluorescence assessment of SP1 after BEZ235 treatment for 24 hrs in A673 **C.** in SKNMC cells **D.** and in HFF **E.** Shown are representative experiments ($n = 3$).

reduction in cell number after treatment, also an effect on cell cycle progression has been noticed in agreement with Manara et al [34]. We hypothesize that the G1 arrest is due to the reduction of EWS/FLI1 levels, whereas further depletion causes cell death in SKNMC cells which are more sensitive than A673 (Supplementary Figure S6C). In agreement with the observed cell cycle arrest, reduced tumor growth was observed when mice were treated with BEZ235 after engraftment of TC71 ES cells [34], with regression induced when combined with vincristine.

Indeed, very little is known about the regulation of EWS/FLI1 transcription and even less about fusion protein turnover. So far, only one study was conducted that implied some possible regulatory regions in the EWSR1 promoter [49]. However, it has been shown previously that inhibition of mTOR by Rapamycin can decrease EWS/FLI1 protein levels [50] similar to what we observed with Rapamycin treatment in A673 cells (Figure 6A).

Inhibition of the PI3K pathway triggered repression of EWS/FLI1 transcription also when the pathway was genetically inhibited by specific siRNA treatment. However, we found that it was not sufficient to deplete one PI3K isoform but simultaneous down regulation of PI3K catalytic subunits α,γ and δ was necessary. This can probably be explained by the lack of mutations in any of these subunits in ES. Thus, the application of more isoform specific inhibitors might be limited and compensatory effects might explain the superior activity of BEZ235 from our panel of inhibitors tested.

Several additional inhibitors were identified from our screen, most notably two γ-secretase inhibitors. However, efforts to genetically verify a potential role of Notch receptors in EWS/FLI1 expression were not successful. This does not exclude a role of the pathway in ES biology as it has been demonstrate already that inhibition of notch can trigger neural differentiation of ES cells [51]. In addition, the most dramatic reduction in cell proliferation was seen with the survivin inhibitor YM155. Indeed, survivin protein and mRNA are found up regulated in ES cells, its expression constitutes a poor

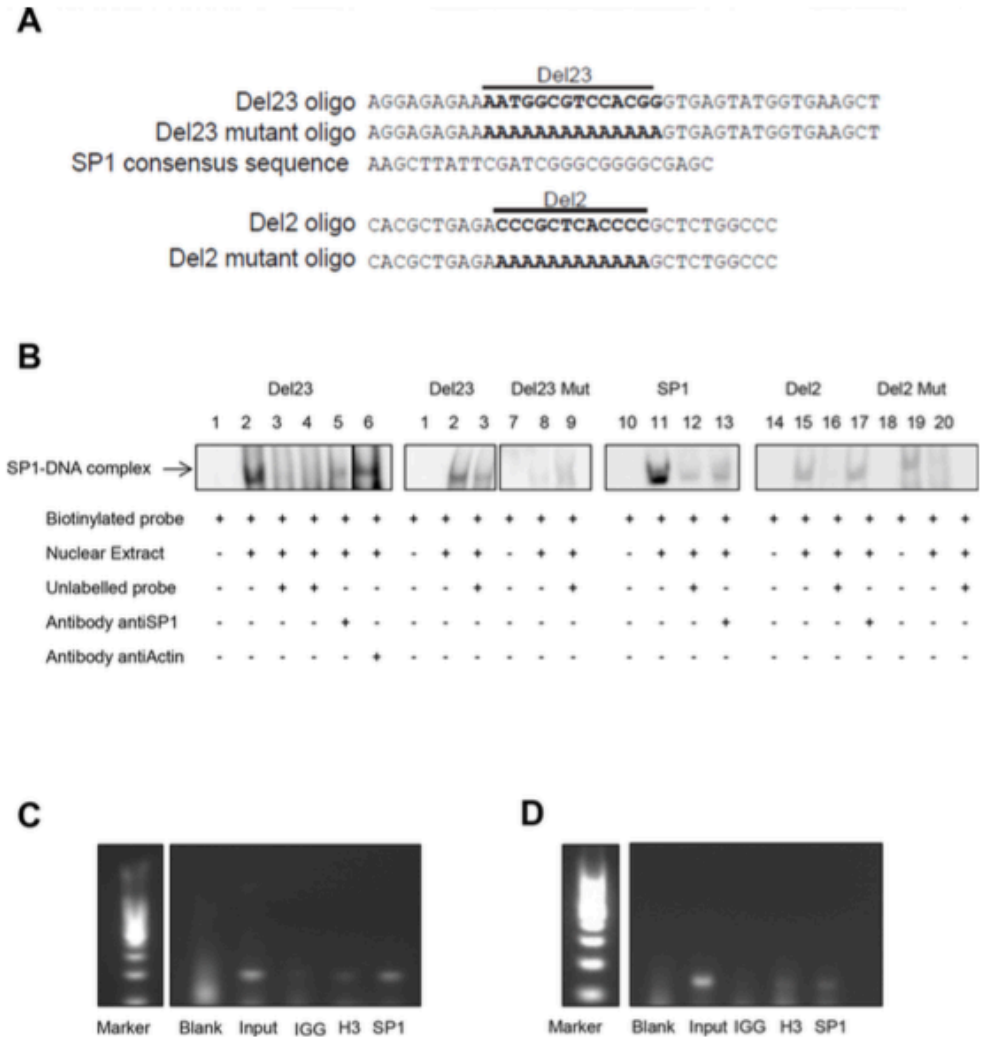

Figure 7: SP1 binds specifically to the Del23 region of the EWSR1 promoter. A. Sequence of Del23, Del23 Mutant, Del2, Del2 Mutant and SP1 oligo. **B.** EMSA assay performed with nuclear extract of A673 cells. In lane 3 competitor oligonucleotide for Del23 was added; in lane 4 and 12 competitor oligonucleotide for SP1. In lane 9 we added Mutant Del23 oligonucleotide, in lane 16 one for Del2 and in lane 20 one for Del2 Mutant. In lane 5, 13 and 17 we added Sp1 antibody. In lane 6 we added Actin antibody. **C.** ChIP assay performed in A673 cells (C) and SKNMC cells **D.** Blank and IGG served as negative control, Input and H3, as positive ones. Shown are representative experiments ($n = 3$).

prognostic marker [52] and genetic knockdown reduced proliferation [53].

We used luciferase reporter assays to characterize for the first time a direct role of the PI3K-AKT-mTOR pathway in transcriptional regulation of the EWS/FLI1 promoter. This effect could be narrowed down to a regulatory element within the promoter, namely the Del23 region, that was bound by the transcription factor SP1 as shown by gel shift and ChIP assays. SP1 is ubiquitously expressed, and binds to GC rich motifs in general. Nevertheless, SP1 did not bind to additional GC-rich regions in the promoter such as Del2. SP1 has recently been described to be activated

via phosphorylation by PI3K Cζ [54] and in addition, SP1 inhibitors, such as Mithramycin, have notable effect on EWS/FLI1 protein activity [55]. Mechanistically, it is still not entirely clear how SP1 activity is regulated by the PI3K pathway. However, we found that treatment specifically reduced SP1 levels, similar to what has been shown previously [56]. However, whether direct phosphorylation at one of the many known sites of SP1 is responsible for this effect, remains to be characterized.

Targeting IGFR1-PI3K-AKT-mTOR signaling has shown promising results in Ewing sarcoma [42, 44, 45, 57]. Our demonstration that inhibition of the pathway directly impairs expression of the fusion protein itself provides additional support for its therapeutic development [58]. Most promising appear to be combinations with other targeted agents that might modulate EWS/FLI1 activity such as YK-4–279 [59] or epigenetic modifiers that can potentially further suppress transcription of the fusion protein. Hence, elucidating the transcriptional regulation of EWS/FLI1 might provide additional molecular targets for this devastating disease.

MATERIALS AND METHODS

Cell lines

Three type 1 (A673, SKNMC, TC71) and two type 2 Ewing cell lines (SKES, RDES) were used. TC71 cells were kindly provided by Prof H. Kovar (St-Anna Children's Hospital, Vienna, Austria) and SKES and RDES by Prof. K.L. Schaefer (Institute of Pathology, Duesseldorf, Germany), A673, HFF and PC3 cells were purchased from the American Type Culture Collection-ATCC (Manassas, VA, USA). Cells were cultivated on 0.2% gelatin coated plates (Sigma-Aldrich, Buchs, Switzerland) in RPMI medium (DMEM for PC3 cells) supplemented with 10% FCS (Sigma-Aldrich), 1% Penicillin/Streptomycin (Thermo Fisher Scientific AG, Reinach, Switzerland), 1% L-glutamine (Bioconcept AG, Allschwil, Switzerland), at 37°C in 5% CO_2.

Screening assay

1.5×10^4 cells were plated in 96-well plates 24 hrs prior to treatment. A library of 153 commercially available targeted inhibitors was acquired from AxonMedchem (Groningen, The Netherlands) and Selleck chemicals LLC (Munich, Germany) (See Supplementary Table S1). Compounds were added to cells in complete RPMI medium at a final concentration of 500 nM for 24 hrs. Lysis and subsequent cDNA synthesis was performed using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies AG, Basel, Switzerland, #600559), followed by quantitative PCR (qPCR). Cell viability was measured in parallel using WST-1 cell proliferation kit (Roche Diagnostics AG, Rotkreuz, Switzerland).

Quantitative PCR

Quantitative PCR (qPCR) was performed under universal cycling conditions on an ABI 7900 instrument using commercially available target probes and mastermix (all from Thermo Fisher Scientific AG). Data were analyzed using SDS 2.2 software (Thermo Fisher Scientific AG). CT values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative expression levels of the target genes were calculated using the ΔΔCT method. All experiments were performed in triplicate and repeated independently at least 3 times. Data analysis was done with the GraphPad prism software (San Diego, CA, USA) and statistical analysis using the Student *t*-test. Commercially available target probes included (Thermo Fisher Scientific AG): EWSR1-FLI1:Hs03024807_ft, FLI1:Hs00956709_m1, SP1:Hs00916521_m1, PHLDA1:Hs00378285_g1, CAV 1: Hs00184697_m1, NKX2.2:Hs00159616_m1, NR0B1: Hs03043658_m1, GAPDH:Hs99999905_m1, PIK3α:Hs0 0907966_m1, PIK3 δ:Hs00192399_m1, PIK3 γ:Hs002770 90_m1, PRKCB:Hs00176998_m1, LOX:Hs00942480_m1, IGFBP3:Hs00365742_g1, STEAP1:Hs00185180_m1.

siRNA treatments

A total of 2×10^6 A673 cells were seeded per 60 mm dish. On the same day, transfection was carried out using LipofectamineRNAi MAX reagent (Thermo Fisher Scientific AG) and 10 nM siRNA of FLI1 (5266), SP1_1 (s13319), SP1_2 (s13320), C-Rel (s11906), NFKB (s9504), YY1 (s224779) PI3K α (s10520), PI3K δ (s10530), PI3K γ (s10532). As a negative control scrambled siRNA n°. 2 (s4390846) was used. All products were purchased from Thermo Fisher Scientific AG. Cells were lysed 48 hrs after silencing and subsequent RNA extraction using RNA easy mini kit (Qiagen Instruments AG, Hombrechtikon, Switzerland) was performed followed by cDNA synthesis with RT kit (Thermo Fisher Scientific AG).

Immunoblotting

Cells were washed twice with PBS and harvested in lysis buffer containing 50 mM NaH_2PO_4 (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM Na3OV4, 5 mM Na-pyrophosphate, 40 nM NaF, 1 mM EGTA supplemented with protease inhibitor cocktail (Complete + 1 mM EDTA, Roche Diagnostics AG). For the preparation of nuclear fraction cells were lysed in 0.1% NP40, washed twice with PBS, centrifuged, and the supernatant discarded. The nuclear pellet was lysed in Roti-Load 1X (Carl Roth GmbH+Co. KG, Karlsruhe, Germany).

Protein concentration was determined by Bradford (Biorad, Reinach, Switzerland) and BCA (Thermo Fisher Scientific AG). 10–30 μg of protein extract was resolved on 4–12% SDS-PAGE and transferred onto nitrocellulose

membrane (Thermo Fisher Scientific AG). Primary antibodies were used as follows: anti-FLI1 monoclonal antibody (MyBioSource LLC, San Diego, CA, USA, 1:1000), anti-PARP rabbit polyclonal antibody (Cell Signalling Technology, Berverly, MA, USA, 1:1000), anti-pAKT antibody (Ser473, Cell Signalling, 1:1000), anti-AKT antibody (Cell Signalling, 1:1000), anti-PHLDA1 antibody (Sigma Aldrich, 1:1000), anti-phospho-mTOR antibody (Ser2448, Cell Signalling, 1:1000), anti-mTOR antibody (Cell Signalling, 1:1000), anti-phospho-S6 Ribosomal protein antibody (Ser235/236, Cell Signalling, 1:2000), anti-S6 Ribosomal protein antibody (Cell Signalling, 1:1000), anti-SP1 antibody (Millipore, Billerica, MA, USA, 1:1000) and anti-β-tubulin I mouse monoclonal antibody (Sigma-Aldrich, dilution 1:40'000). After incubation with the appropriate secondary peroxidase-conjugated antibodies (1:1'000), detection was performed with the ECL chemiluminescence reagent (Amersham Biosciences, Freiburg, Germany).

Luciferase assays

The promoter region of EWS/FLI1 (Ref. sequence NM_013986) covering 2.3kb (position -2239/+67 relative to the transcription initiation site) was cloned in pGL4.19 luciferase vector (Promega AG, Madison, WI, USA) using Infusion HD cloning kit (Clontech Laboratories, Inc Mountain View, CA,USA). With the same approach several deletion constructs of the EWS/FLI1 promoter were made, namely -1708/+67 -1277/+67, -774/+67, -275/+67. Using a site directed mutagenesis kit (Thermo Fisher Scientific AG), 24 additional deletion mutants of the -275/+67 construct were made (see Supplementary Table S2 for a detailed list of the plasmids). All constructs were verified by sequencing.

2×10^4 A673 cells per well were plated in 96-well plate and transfected 24 hrs later using Jet Prime (Polyplus Transfection, Strasbourg, France) with 100 ng of reporter construct, or empty vector (pGL4.19) as a negative control. For normalization, cells were co-transfected with 10 ng of a renilla luciferase plasmid. After 24 hrs cells were treated with 50 nM BEZ235 or DMSO. 48 hrs post transfection they were lysed and assayed for luciferase activity using the Dual Glo luciferase reporter system (Promega AG).

Electrophoretic mobility shift assays

1×10^7 cells were plated, washed once with PBS 24 hrs later and lysed in Buffer A (10 mM Hepes pH 7.9, 10 mMKCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) containing 10% NP40. The nuclear pellet was transferred to Buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM DTT, 1x complete Mini-Roche tablet). 40 µg of nuclear extract, 100 nM of biotinylated oligo, 4 µM of unlabelled probe, 1 µL of anti-SP1 (Millipore) or anti Actin antibodies (Cell Signalling) were mixed. Oligonucleotides were ordered from Microsynth AG, Balgach, Switzerland) and annealed with Annealing Buffer (10 mM Tris, 1 mM EDTA, 50 mM NaCl pH 8.0):

Del23_Forward: AGGAGAGA**AAATGGCGTCCACGG**GTGATATGGTGAAGCT (biotin);

Del23 mutant_Forward: AGGAGAGA**AAAAAAAAAAAAAAA**GTGAGTATGGTGAAGCT (biotin);

Del2_Forward: CACGCTGAGA**CCCGCTCACCCC**GCTCTGGCCC (biotin);

Del23 mutant_Forward: CACGCTGAGA**AAAAAAAAAAAAAA**GCTCTGGCCC (biotin);

SP1_Forward: AAGCTTATTCGATCGGGCGGGGCGAGC (biotin).

ChIP assay

1×10^7 cells were plated and cross-linked after 24 hrs with 1% formaldehyde (Thermo Fisher Scientific AG) for 5–10 minutes at RT. Formaldehyde was quenched by adding 125 mM Glycine (Sigma-Aldrich) for 5 minutes. Cells were collected, washed twice with cold PBS and lysed in IP buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% NP-40, 1.0% Triton X-100). The crude extract was washed twice with IP buffer and sonicated 15 times for 20s [60]. Samples were incubated overnight at 4°C with the anti SP1 (Millipore), anti H3 (Cell Signalling) or anti IgG (Cell Signalling) antibodies and then immunoprecipitated (Active Motif kit). Primers (Microsynth AG) were as follows: CGAGTAAGCGGTGGTTCATC (forward).

Immunofluorescence

Cells were washed once with PBS, fixed with 4% PFA, washed again with PBS and then with PBS-0.1% TritonX. Hereafter, they were incubated overnight with the primary antibody - FLI1 (1:50) (Sigma-Aldrich), SP1 (1:500) (Millipore), - diluted in PBS-0.1% TritonX with 4% Horse Serum (Sigma-Aldrich). Afterwards, cells were washed and incubated for 1 hr at room temperature with the secondary antibody (1:500) diluted in PBS - 4% horse serum (Sigma-Aldrich). Cells were washed twice with PBS and once with distilled water; one drop of Dapi (Vechtashield H-1200, Vector Laboratories, Inc. Burlingame, CA, USA) added and analyzed with a Zeiss inverted microscope.

Casp3/7 assay

4×10^3 A673 and SKNMC cells, were plated in a 384 well plate previously coated with 0.2% gelatin. After 24 hrs, cells were treated with 500 nM BEZ235, 1 µM Staurosporin, 100 nM Nocodazole or DMSO as controls. 24 hrs after treatment Caspase 3/7 reagent (Promega AG) was added in each well and luminescence was measured.

FACS analysis

Treated cells were washed with PBS, collected, fixed with 70% Ethanol for 2 hrs on ice and stained with PI solution (20 µg/ml PI (Sigma-Aldrich), PBS- 0.1% TritonX 200 µg/ml RNAse A for measurement with a FACS Canto. Data were analyzed using Flow Jo program (Flow Jo LLC., Ashland, OR, USA).

GRANT SUPPORT

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CONFLICTS OF INTEREST

There are no potential conflicts of interest.

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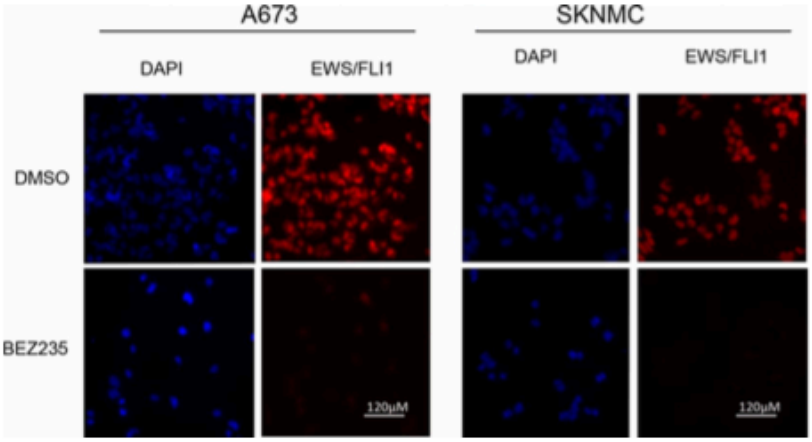
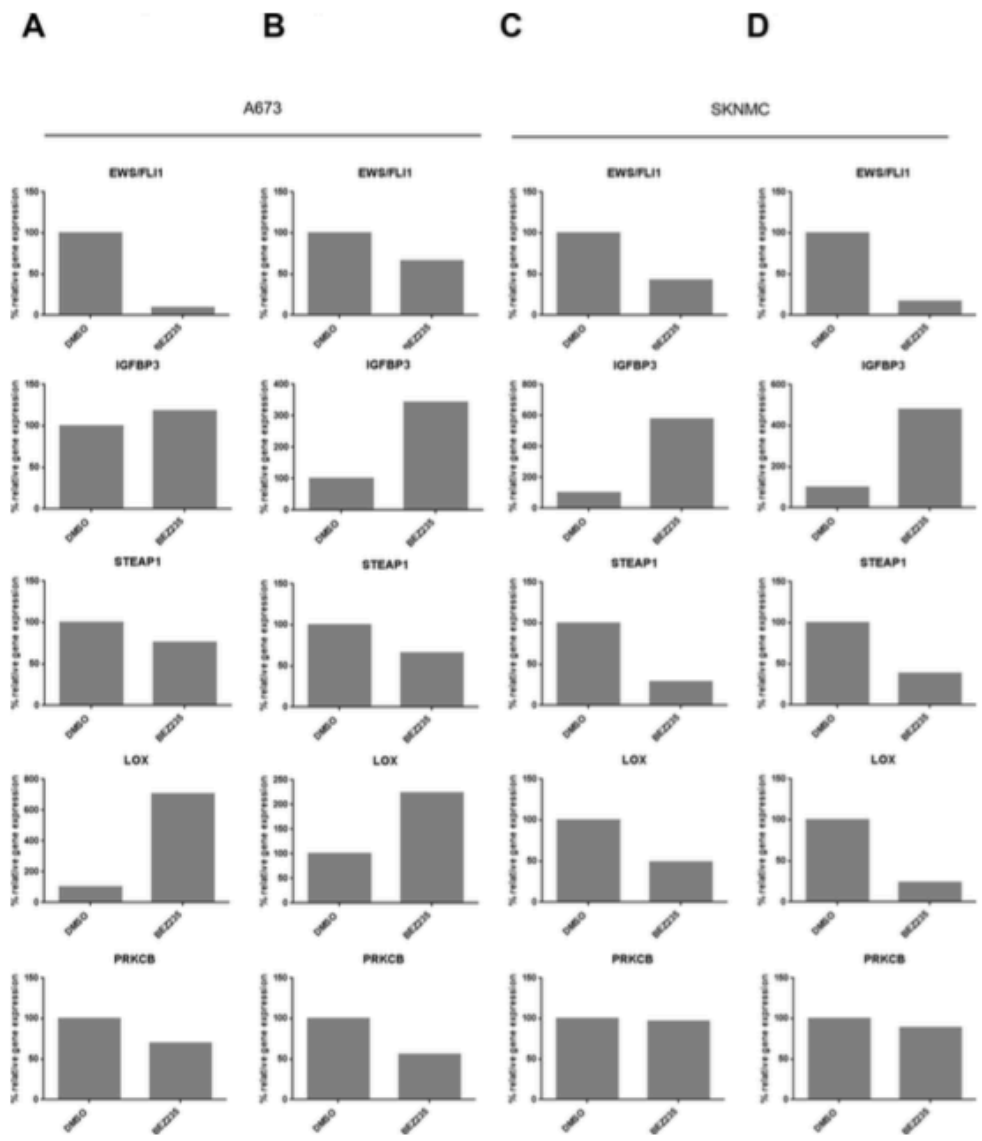
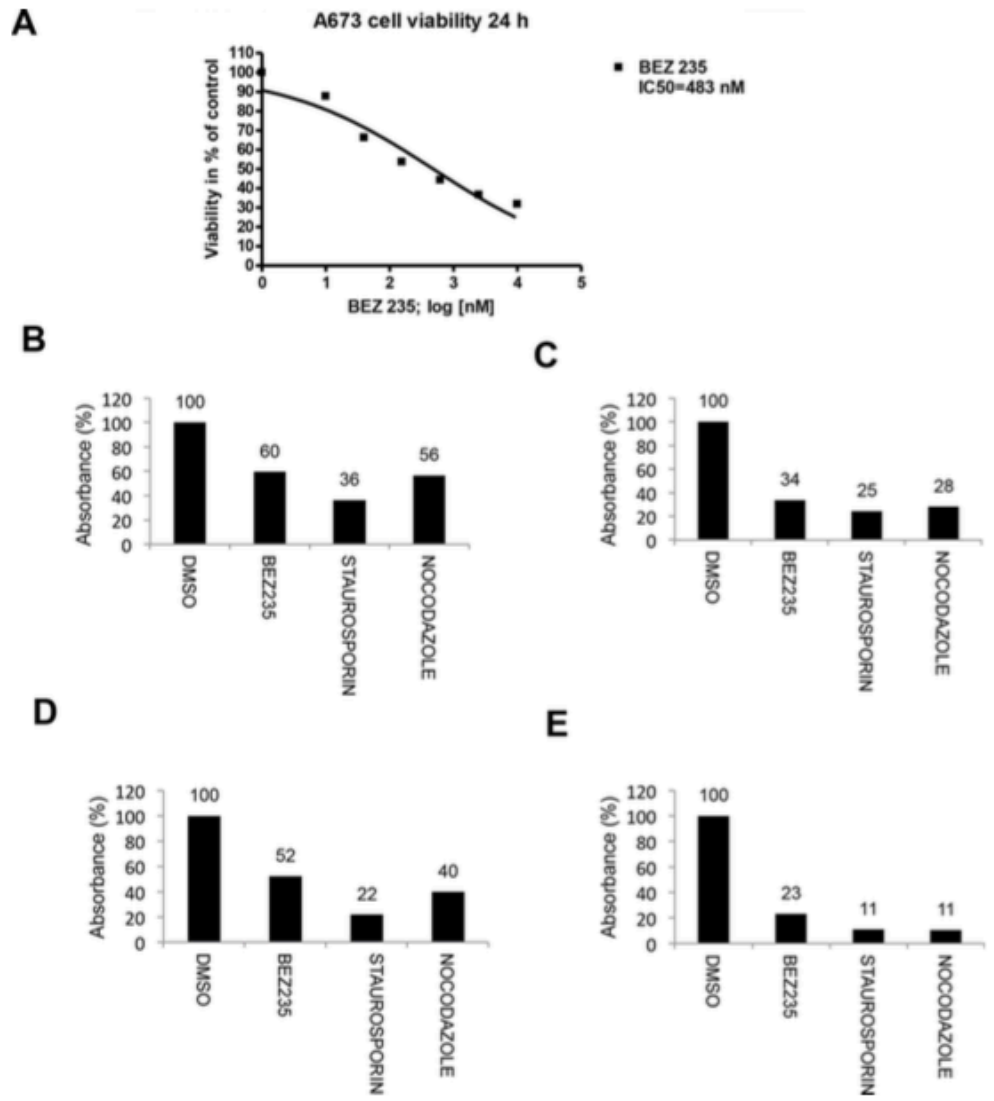
SUPPLEMENTARY FIGURES AND TABLES



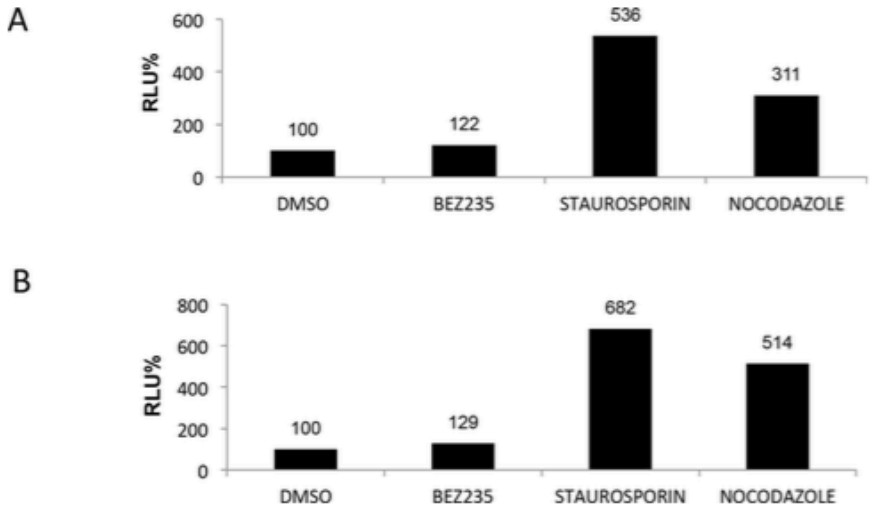
Supplementary Figure S1: ***BEZ235 treatment affects EWS/FLI1 level.*** Representative experiment ($n = 3$) of immunofluorescence assessment of EWS/FLI1 after BEZ235 treatment for 24 hrs in A673 and in SKNMC cells.



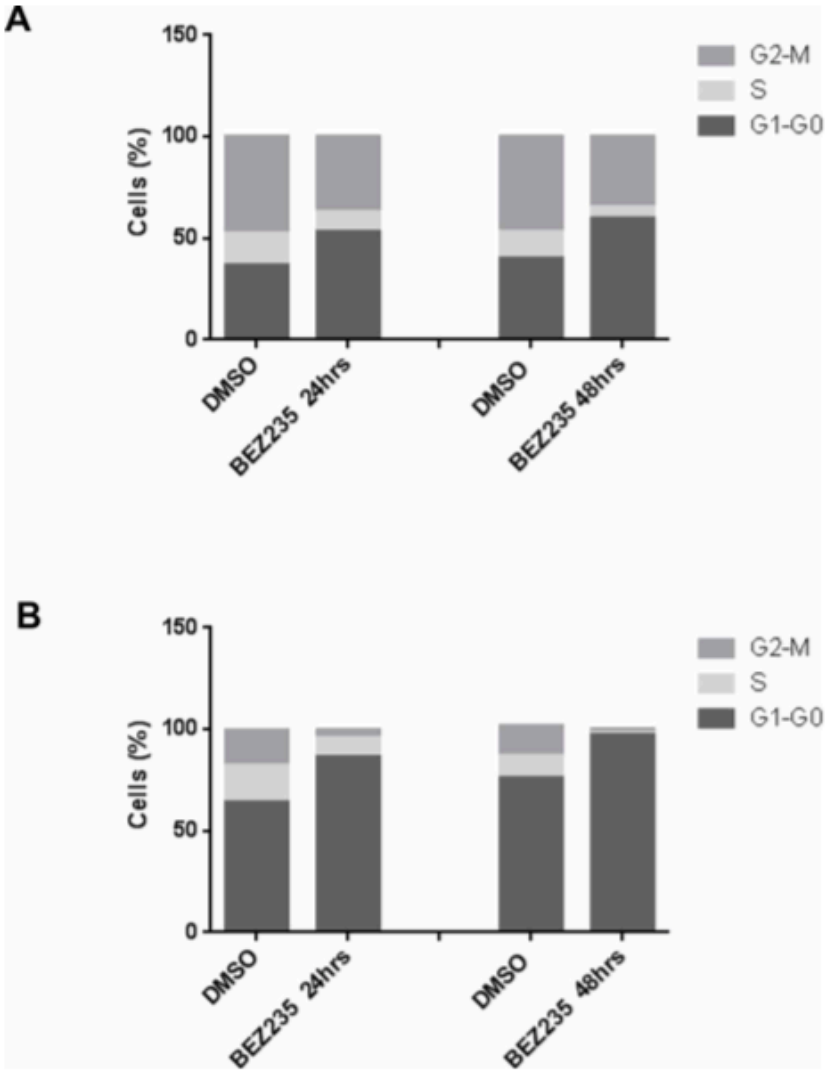
Supplementary Figure S2: ***BEZ235 treatment affects EWS/FLI1 gene expression and its target genes.*** Gene expression level in percentage of EWS/FLI1 and its repressed (LOX and IGFBP3) and activated (PRKCB and STEAP1) target genes after BEZ235 treatment in A673 **A, B.** and in SKNMC cells **C, D.** for 24 (A,C) and 48 hrs (B,D). Shown is a representative experiment ($n = 3$).



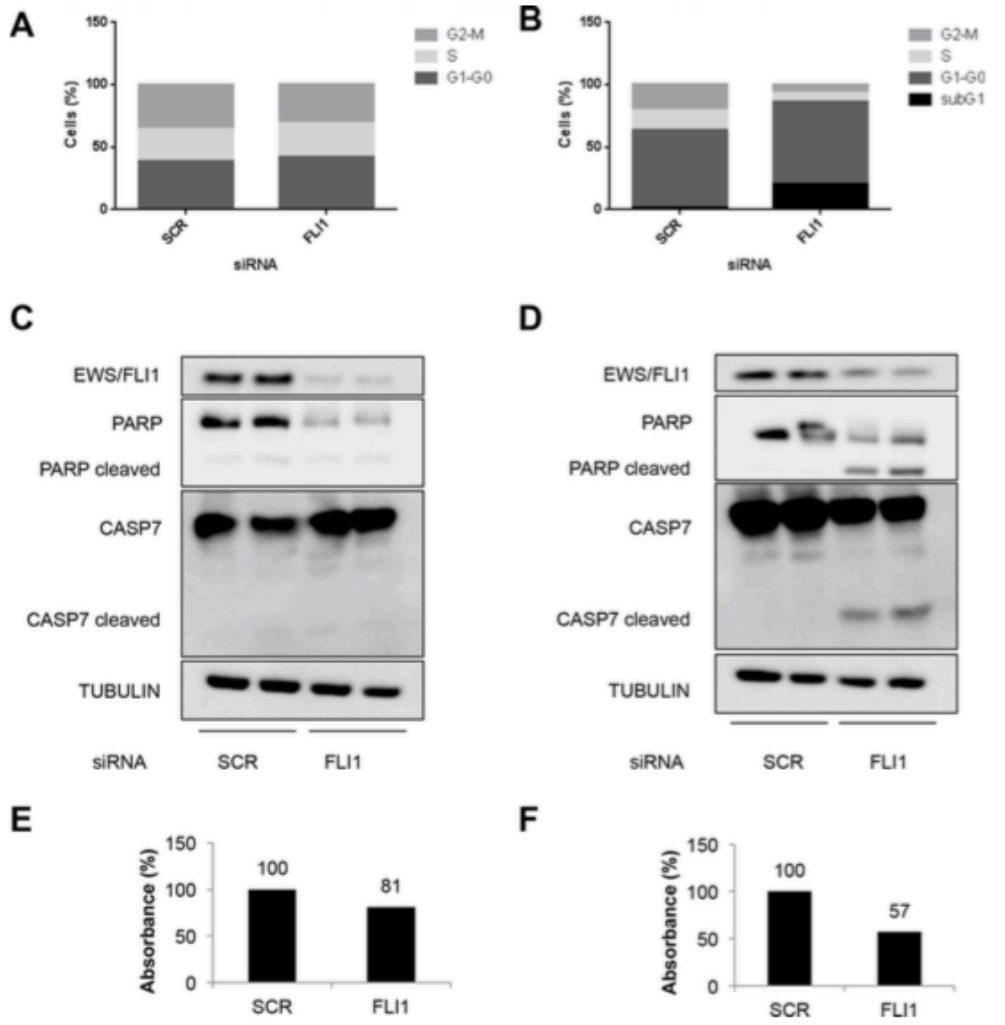
Supplementary Figure S3: A. A673 cells viability after 24 hrs treatment with titrating concentration of BEZ235. Absorbance in percentage compared to DMSO treated control after drug treatment for 24 and 48 hrs in A673 **B.** and **C.** and SKNMC cells **D.** and **E.** Bars represent mean values of 8 biological replicates analysed in three technical replicates each ($n = 3$; SE < 0.01).



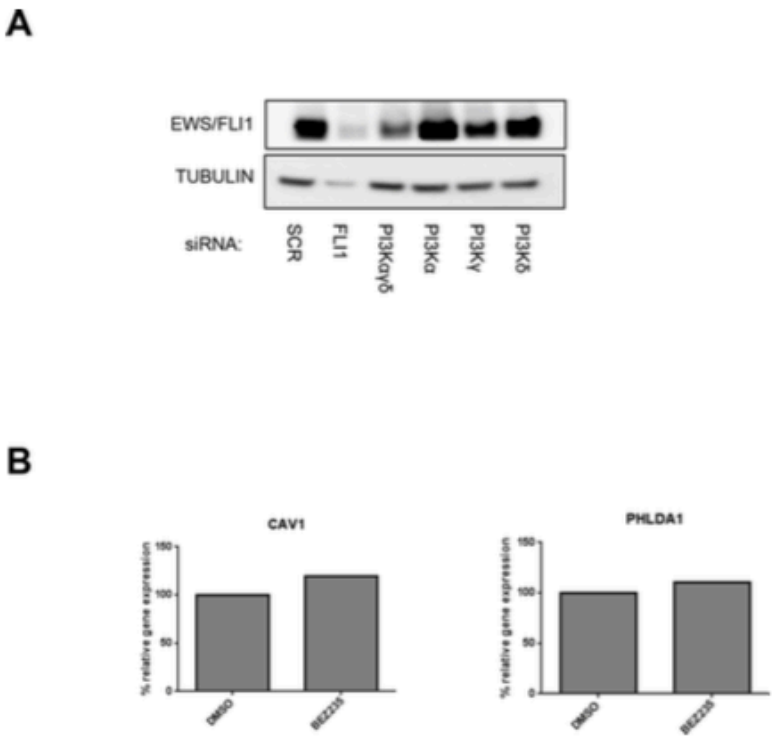
Supplementary Figure S4: ***CASPASE 3/7 ACTIVITY.*** Caspase 3/7 activity measured after 24 hrs treatment with 500 nM BEZ235, 1 μM Staurosporin and 100 nM Nocodazole in A673 **A.** and SKNMC cells **B.** Bars represent mean values expressed as relative light unit (RLU) in percentage of DMSO treated control of 6 biological replicate analysed in three technical replicates each ($n = 3$; SE < 0.01).



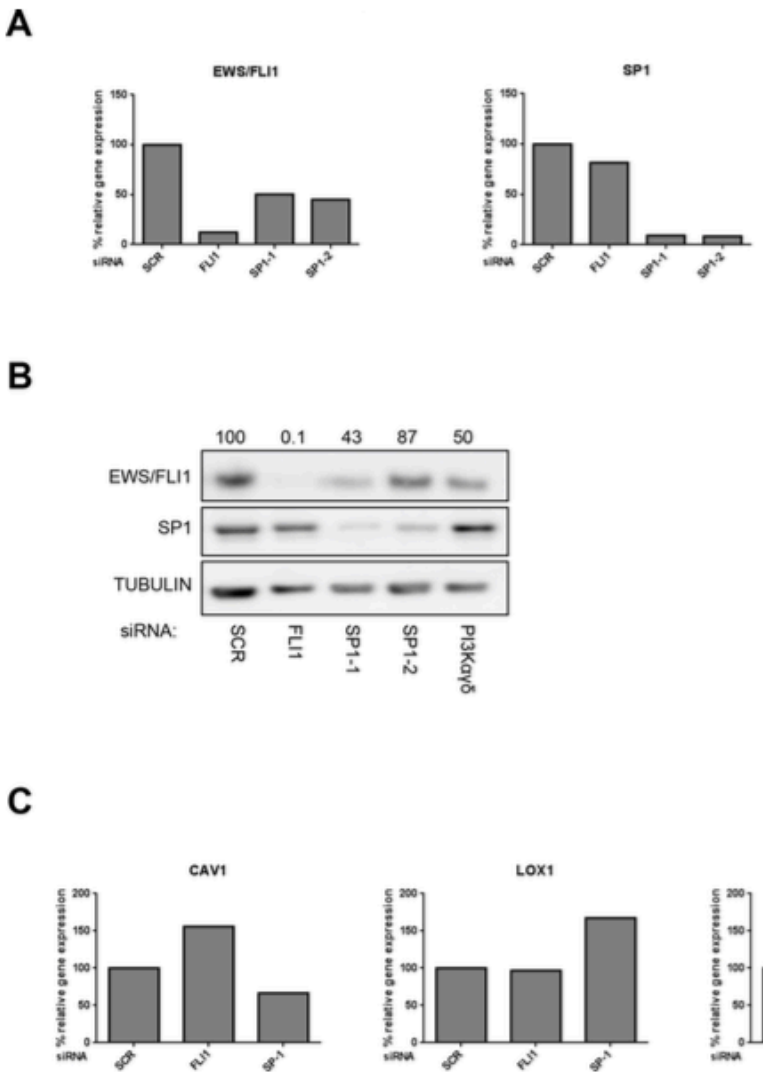
Supplementary Figure S5: ***Cell cycle progression upon BEZ235 treatment.*** Cell cycle analysis after 24 and 48 hrs treatment with 500 nM BEZ235 compared to DMSO treated control in A673 **A.** and SKNMC **B.** Shown is a representative experiment ($n = 3$).



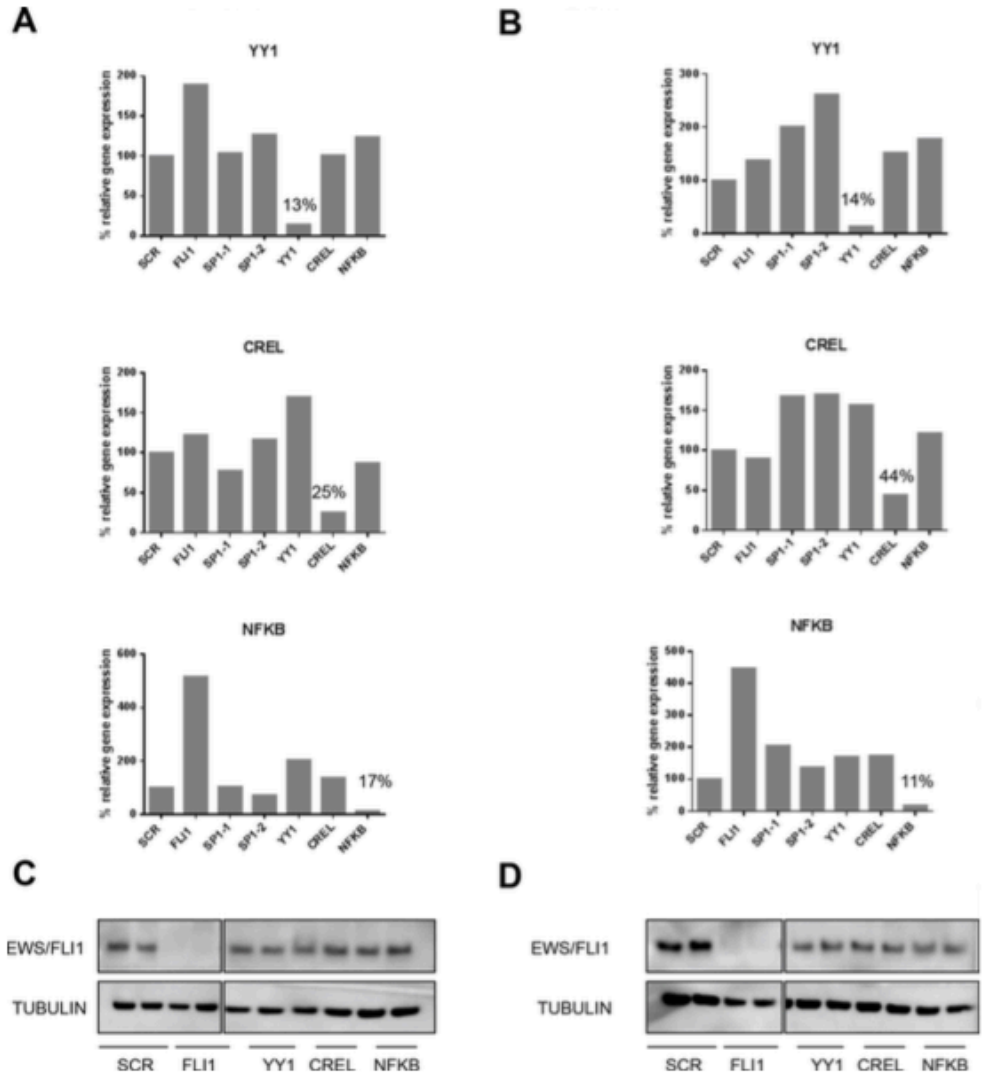
Supplementary Figure S6: ***Cell cycle progression after silencing of EWS/FLI1.*** Cell cycle analysis upon EWS/FLI1 depletion for 48 hrs in A673 **A.** and SKNMC cells **B.** Protein level measured by western blot of EWS/FLI1, PARP, CASP7 and TUBULIN in A673 **C.** and SKNMC cells **D.** after silencing of EWS/FLI1 in biological duplicates. **E.** Absorbance in percentage of viable cells upon depletion of EWS/FLI1 in A673 and SKNMC cells **F.** compared to Scr control . Shown are representative experiments ($n = 3$).



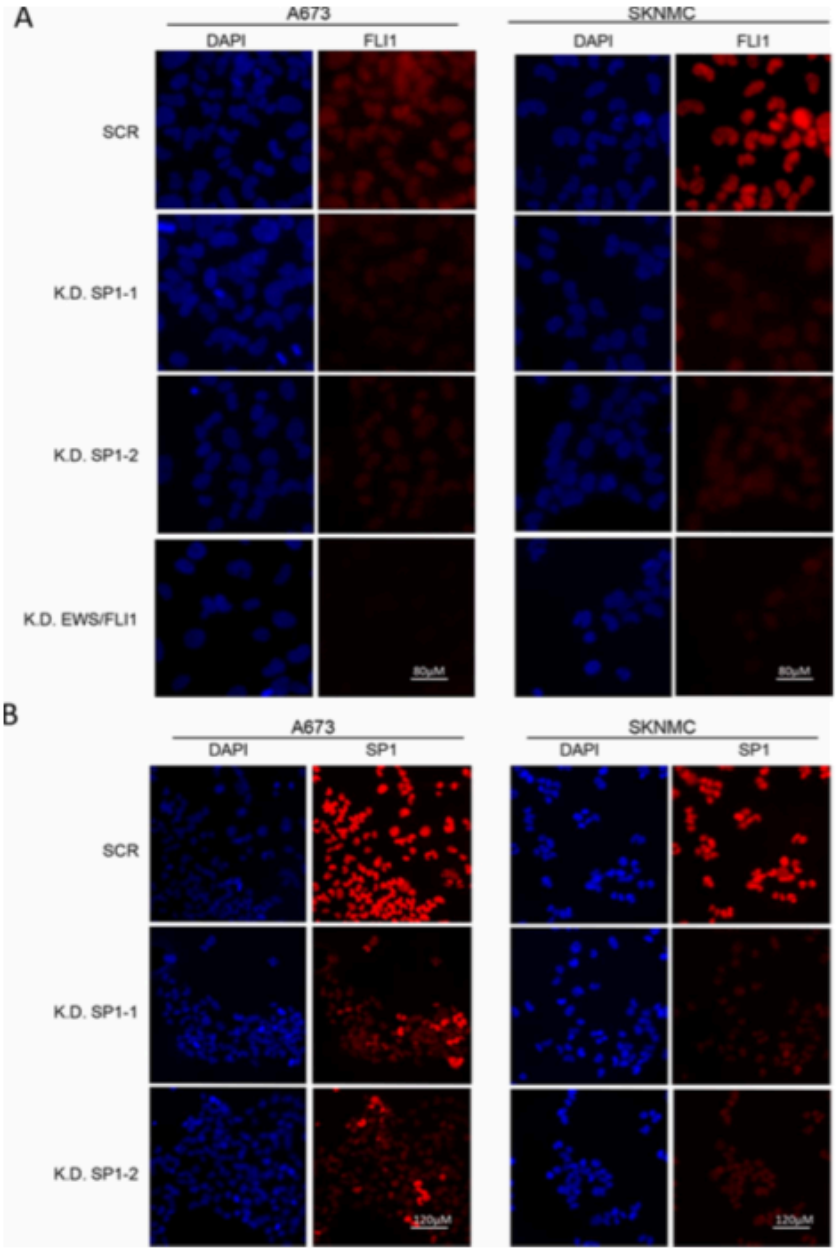
Supplementary Figure S7: ***Effects of PI3K pathway inhibition on EWS/FLI1 and ist target genes.*** **A.** siRNA mediated knockdown of PI3K components for 48 hrs in order to determine EWS/FLI1 level in SKNMC cells. **B.** mRNA level of EWS/FLI1 target genes expressed endogenuously in PC3 cells after BEZ235 treatment for 24 hrs. Shown are representative experiments ($n = 3$).



Supplementary Figure S8: ***Effects of SP1 silencing on EWS/FLI1 level and ist target genes.*** **A.** siRNA mediated knockdown of SP1 for 48 hrs in order to determine mRNA expression of EWS/FLI1 , and SP1 (via qRT-PCR) in SKNMC cells. **B.** Protein level assesment of EWS/FLI1 via western blot after sliencing of SP1 or PI3Kαγδ in SKNMC cells. **C.** mRNA level of EWS/FLI1 target genes expressed endogenuously in PC3 cells after silencing of SP1 for 48 hrs. Shown are representative experiments ($n = 3$).



Supplementary Figure S9: ***Validation of the knock down of transcription factors candidates for the Del23 region and its effect on EWS/FLI1.*** Gene expression data after reverse knock down candidates for Del23 region in SKNMC **A.** and A673 **B.** EWS/FLI1 protein level after silencing for 48 hrs of the candidate transcription factors by siRNA in SKNMC cells **C.** and A673 **D.** Shown are representative experiments ($n = 3$).



Supplementary Figure S10: ***SP1 knock down affects EWS/FLI1 level.*** Immunofluorescence assessment of EWS/FLI1 **A.** and SP1 **B.** after reverse knock down of SP1 in A673 and SKNMC cells. Exposure times were kept constant for all samples.

Supplementary Table S1: List of targeted inhibitors used in the screening

No.	Name	Target
1	[Ala92]-p16 (84–103)	Cdk inhibitor
2	17-AAG	Hsp90 inhibitor
3	2-Deoxy-D-glucose	Glycolysis inhibitor
4	3-MA	Vps34 inhibitor (class III PI3K)
5	A 769662	AMPK activator
6	ABT-102	TRVP1 antagonist
7	ABT-737	BCL-2, BCL-xl inhibitor
8	AEG 3482	JNK inhibitor
9	AG 013736 - Axitinib	VEGFR inhibitor
10	AMD3100	CXCR4 inhibitor
11	AMN 107 (Nilotinib)	BCR-ABL inhibitor
12	Apratastat	TACE/MMP inhibitor
13	AS 252424	PI3K p110 gamma inhibitor
14	AT9283	Aurora kinases Inhibitor
15	Atazanivir	Protease inhibitor
16	AZD 2281 - Olaparib	PARP inhibitor
17	AZD 7762	CHK inhibitor
18	AZD1152-HQPA	Aurora B inhibitor
19	BACE 1 inhibitor	Beta Secretase inhibitor
20	Bax inhibitor peptide P5	Bcl-2 Protein Family Inhibitor
21	Bax inhibitor peptide V5	Bcl-2 Protein Family Inhibitor
22	Bax inhibitor peptide,negative control	Bcl-2 Protein Family Inhibitor
23	BAY 61–3606	Syk inhibitor
24	BI 2536	PLK-1 inhibitor
25	BMS 189961	RAR gamma agonist
26	BMS 270394	RAR gamma agonist
27	BMS-345541	IκB inhibitor
28	Bosutinib (SKI 606)	BCR-ABL/SRC inhibitor
29	Butabindide	TPPII inhibitor
30	BX 795	PDK1/TBK1 inhibitor
31	BX 912	PDK1 inhibitor
32	BZ - Gamma Secretase inhibitor BZ	Gamma Secretase inhibitor
33	Cediranib	VEGFR inhibitor
34	CH 55	RAR alpha/beta agonist

(*Continued*)

No.	Name	Target
35	Chir98014	GSK-3 inhibitor
36	CI-1033 - Canertinib	EGFR inhibitor
37	Combretastatin-A4 (CA-4)	Inhibitor of tubulin polymerization
38	Compound C	AMPK inhibitor
39	CP 690550	JAK3 inhibitor
40	CT 99021 - CHIR 99021	GSK-3 inhibitor
41	Cyclopamine	Hedgehog Pathway Inhibitor
42	Cyclosporine	Immunosupressant
43	DAPT	Gamma Secretase inhibitor
44	Dasatinib	BCR-ABL/SRC inhibitor
45	DBZ	Gamma Secretase inhibitor
46	Deguelin	Anticancer agent
47	DM 3189	BMP inhibitor
48	Doramapimod	p38 MAPK inhibitor
49	DR 2313	PARP inhibitor
50	FK 866	NAPRT1 inhibitor, anti-cancer agent
51	GDC 0879	B-Raf inhibitor
52	GDC 0941	PI3K inhibitor
53	GDC-0449	Hedgehog Pathway Inhibitor
54	Gefitinib	EGFR inhibitor
55	GSK 269962A	ROCK1 inhibitor
56	GW 441756	TrkA inhibitor
57	GW 786034	VEGFR/KIT/PDGFR inhibitor
58	GW 843682X	PLK inhibitor
59	Honokiol	pAkt, scr, p44/42 MAPK
60	HU-308	CB2 agonist
61	IGC-001	Beta-Catenin inhibitor
62	Imatinib	BCR-ABL inhibitor
63	JAK inhibitor I	JAKs inhibitor
64	JIP-1 (153–163)	JNK Inhibitor
65	JWH 018	CB2 agonist
66	JWH 073	CB1/2 agonist
67	JWH 133	CB2 agonist
68	Ko 143	BCRP inhibitor
69	KU-55933	ATM inhibitor
70	L-685,485	Gamma Secretase inhibitor

(*Continued*)

No.	Name	Target
71	L-aminoadipic acid	Glutamine synthase inhibitor
72	Lamotrigine	Glutamate antagonist
73	Lapatinib	EGFR/ErbB-2 inhibitor
74	LE-135	RAR beta antagonist
75	LY 2157299	TGF beta receptor inhibitor
76	LY 294002	PI3K inhibitor
77	Masitinib mesylate	KIT/PDGFR inhibitor
78	MK 1775	Wee1 inhibitor
79	MK-2206	Akt inhibitor
80	MLN8237	Aurora kinase inhibitor
81	Myoseverin	
82	Na-Butyrate	HDAC inhibitor
83	NEC-1	Necroptosis/RIPK inhibitor
84	NF-kB Inhibitor	NF-kB Inhibitor
85	Nocodazole	Cell cycle G2/M inhibitor
86	NSC 348884	Nucleophosmin inhibitor
87	NSC 625987	CDK4 inhibitor
88	NU 1025	PARP inhibitor
89	NU 7441	DNA-PK inhibitor
90	NVP-AEW514	IGF-1R inhibitor
91	NVP-AUY922	HSP90 inhibitor
92	NVP-BAG956	PI3K/PDK1 inhibitor
93	NVP-BEZ235	PI3K/mTOC inhibitor
94	NVP-BGJ398	FGF-R inhibitor
95	NVP-BKM120	PI3K, not mTOC inhibitor
96	NVP-BSK805	JAK2 inhibitor
97	NVP-TAE684	ALK inhibitor
98	NVP-TKI258	FGF-R inhibitor
99	Obatoclax	Bcl-2 inhibitor
100	OSI 774 - Erlotininb	EGFR inhibitor
101	OSI-027	mTORC1/2 inhibitor
102	Palmitoylethanolamide	Endocannabinoid
103	PD 0325901	MEK inhibitor
104	PD 166793	MMP inhibitor
105	PD 169316	p38 MAPK inhibitor
106	PD 180970	Src kinase inhibitor
107	PD 184352	MEK 1 inhibitor

(*Continued*)

No.	Name	Target
108	PD 98059	MEK inhibitor
109	PD150606	Calpain inhibitor
110	PF-00356231	MMP-12 inhibitor
111	PHA-739358(Danusertib)	Aurora kinases,Bcr-Abl and FGFR inhibitor
112	PI 103	Class I PI3K inhibitor
113	piceatannol	Syk inhibitor
114	PIK 75	PI3K p110 alpha inhibitor
115	PIK 90	PI3K p110 alpha inhibitor
116	pimecrolimus	Calcineurin inhibitor
117	PLX 4720	B-Raf inhibitor
118	PP2	Src inhibitor
119	PP242	mTORC1/2 inhibitor
120	PU-H71	HSP90 inhibitor
121	Roscovitine/Celiciblib	Cdk inhibitor
122	Ruboxistaurin (LY333531)	PKC beta inhibitor
123	S31–201	Stat3 Inhibitor
124	Saracatinib	Src and Abl inhibitor
125	SB 202190	p38 MAPK inhibitor
126	SB 203580	p38 MAPK inhibitor
127	SB 216763	GSK-3 inhibitor
128	SB 431542	TGF beta receptor inhibitor
129	Scriptaid	HDAC inhibitor
130	SD 169	p38 MAPK inhibitor
131	SD 208	TGF-betaR receptor 1 inhibitor
132	SL 327	MEK1/2 inhibitor
133	SNS-314	Aurora kinase inhibitor
134	Sorafenib (BAY 43–9006)	Raf/Mek/Erk inhibitor
135	Stobadine	Antioxidant
136	SU 6656	Src kinase inhibitor
137	SU11274	c-Met inhibitor
138	Sunitinib - SU 11248	Multiple RTK inhibitor
139	Tacrolimus	Calcineurin inhibitor
140	Tandutinib	FLT3 inhibitor
141	TG101348	JAK2 (Flt3) inhibitor
142	TGX 221	PI3K p110 beta inhibitor
143	Tiplaxtinin	PAI-1 inhibitor
144	TW-37	Bcl-2 protein family inhibitor

(*Continued*)

No.	Name	Target
145	Tyrphostin AG 490	JAK2 inhibitor
146	U 73122	Phospholipase C inhibitor
147	Vandetanib	VEGFR/EGFR Inhibitor
148	Velcade	Proteasome inhibitor
149	Vorinostat/SAHA	HDAC inhibitor
150	VPA	HDAC inhibitor
151	xav-939	Wnt/beta-catenin signal transd. inhibitor
152	XL228	Multiple Tyr-kinase inhib. (IGF1-R, Bcr-Abl)
153	Y-27632	p160 ROCK inhibitor

Supplementary Table S2: Deletion constructs of the 2.3kb EWS/FLI1 promoter construct

Name of the construct	Position of the deletion relative to the transcription initiation site
Del1	−214/−203
Del2	−202/−191
Del3	−190/−179
Del4	−178/−167
Del5	−166/−155
Del6	−154/−143
Del7	−142/−131
Del8	−130/−119
Del9	−118/−107
Del10	−106/−95
Del11	−94/−83
Del12	−82/−71
Del13	−70/−59
Del14	−58/−47
Del15	−46/−35
Del16	−34/−23
Del17	−22/−11
Del18	−10/+2
Del19	+3/+14
Del20	+15/+26
Del21	+27/+40
Del22	+41/+54
Del23	+55/+68
Del24	+69/+80

5. 2 Targeting the EWS-ETS transcriptional program by BET bromodomain inhibition in Ewing sarcoma

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Contribution: TH, OS, J.C-W., GHSR and I performed experiments. I further performed drug treatments, Cell cycle analysis and Caspase 3/7 assay staining (Fig. 1B,C/ Fig. 3 C,D/ Suppl. Fig. 2A).

BS, FKN, SB and GHSR initiated the project. TH and GHSR wrote the paper.

Contribution: TH, OS, J.C-W., GHSR and I performed experiments. TH, BS, FN, GHSR and I analyzed data. BS, FKN, SB and GHSR initiated the project. TH and GHSR wrote the paper.

Targeting the EWS-ETS transcriptional program by BET bromodomain inhibition in Ewing sarcoma

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ABSTRACT

Ewing sarcomas (ES) are highly malignant bone or soft tissue tumors. Genetically, ES are defined by balanced chromosomal *EWS/ETS* translocations, which give rise to chimeric proteins (EWS-ETS) that generate an oncogenic transcriptional program associated with altered epigenetic marks throughout the genome. By use of an inhibitor (JQ1) blocking BET bromodomain binding proteins (BRDs) we strikingly observed a strong down-regulation of the predominant EWS-ETS protein EWS-FLI1 in a dose dependent manner. This was further enhanced by co-treatment with an inhibitor of the PI3K pathway. Microarray analysis further revealed JQ1 treatment to block a typical ES associated expression program. The effect on this expression program was mimicked by RNA interference with BRD3 or BRD4 expression, indicating that the EWS-FLI1 mediated expression profile is at least in part mediated via such epigenetic readers. Consequently, contact dependent and independent proliferation of different ES lines was strongly inhibited. Mechanistically, treatment of ES resulted in a partial arrest of the cell cycle as well as induction of apoptosis. Tumor development was suppressed dose dependently in a xeno-transplant model in immune deficient mice, overall indicating that ES may be susceptible to treatment with epigenetic inhibitors blocking BET bromodomain activity and the associated pathognomonic EWS-ETS transcriptional program.

INTRODUCTION

Ewing sarcoma (ES) is a highly malignant bone and soft tissue neoplasia of still enigmatic histogenesis with a prominent *stemness* phenotype [1, 2]. Histogenesis may be endothelial, neuroectodermal [3-5] or osteo-chondrogenic [6, 7]. ES are characterized by early metastasis into lung and bone tissues. Metastasis is commonly haematogenous and related to *stemness* [1, 4, 8]. Even though prognosis for ES patients has markedly improved with the development of multimodal therapeutic approaches, the survival rate of patients with advanced, multifocal disease is still associated with fatal outcome [9-11]. Especially multifocal bone or bone marrow disease and the development of metastases in bones are catastrophic events in the clinical course of ES patients [9, 12]. Genetically, ES is defined by specific balanced chromosomal *EWS/ETS* translocations which give rise to oncogenic chimeric proteins, the most common being EWS-FLI1 as a consequence of the t(11;22)(q24;q12)

translocation [13-15]. Other contributing somatic mutations involved in disease development have only been observed at low frequency [16-19].

Thus, cancer in children is not solely a genetic disease and can neither be understood nor cured presumably without epigenetics. We previously identified the histone methyl-transferase Enhancer of Zeste, Drosophila, Homolog 2 (EZH2), the enzymatic subunit of the polycomb PRC2 complex, to be over-expressed and regulated as a downstream event via EWS-FLI1 in ES. RNA interference of EZH2 suppressed tumor development and metastasis *in vivo* and microarray analysis of EZH2 knock down revealed an EZH2-maintained, undifferentiated, reversible phenotype in ES [1]. EZH2 suppression resulted in a generalized loss of H3K27me3 as well as increase in H3 acetylation. ChIP-Chip assays for H3K27me3 verified such genes that had specifically lost H3K27me3 upon EZH2 silencing [8], suggesting that malignant *stemness* features are preserved via epigenetic mechanisms. Recent results further indicate that EWS-ETS proteins not only deregulate components of the epigenetic machinery in ES [1], but in addition create specific epigenetic marks [20, 21] that might be addressed by epigenetic therapy.

BET proteins (BRD2, BRD3, BRD4, and the testis-specific BRDT) are bromodomain (BRD) containing proteins that belong to the bromo and extraterminal (BET) subset of BRD proteins. They are nuclear proteins that carry 2 bromodomains and an additional ET domain, and are implicated in chromatin interactions [22]. They seem to associate with transcription complexes and with acetylated chromatin [23]. Specific inhibitors of BET proteins such as I-BET151 or JQ1 resulted in displacement of BRDs from chromatin and inhibition of transcription at key genes such as BCL2, MYC, and CDK6 [23]. Initially it was shown that JQ1 could block the growth of a rare, aggressive form of human squamous carcinoma with BRD4-NUT translocation [24] as well as of MYC transformed multiple myeloma [25]. Effectivity of JQ1 and inhibition of C-MYC or N-MYC was also demonstrated for AML [26] or neuroblastoma [27], respectively.

In addition to BET inhibitors, also enhanced activity of the phosphoinositide 3-kinase (PI3K) pathway has been linked to MYC turnover [28] and thereby might potentially enhance the activity of BET inhibitors. Indeed, PI3K inhibition has been suggested as therapeutic option in ES before [29] and recent evidence suggests that the pathway can modulate expression of the EWS-FLI1 fusion protein itself [30]. By use of the BET bromodomain inhibitor JQ1 we significantly blocked proliferation and *in vivo* tumor growth of different ES lines and strikingly observed a strong down-regulation of the pathognomonic EWS-FLI1 protein. Subsequent analysis revealed that JQ1 treatment blocked an ES specific expression program and enhanced apoptosis of treated cell lines.

RESULTS

JQ1 blocks EWS-FLI1 expression in ES

In a previous microarray analysis we identified the proto-oncogene MYC as being persistently up-regulated in ES (Supplementary Figure S1). To analyze the relevance of its expression, we employed the potent BET bromodomain inhibitor JQ1 and the PI3K inhibitor BEZ235 as possible pathways regulating MYC expression in ES cells and compared their impact to such on mesenchymal stem cells (MSCs). None of the analyzed ES cells showed any down-regulation of MYC expression after treatment with different concentrations of JQ1 (Figure 1A, Top) while in contrast MSCs showed an up to 65% down-regulation after 5μM JQ1 treatment in VH54.2 cells (Figure 1A, Bottom). Similarly, also BEZ235 treatment revealed no influence on MYC expression in ES (data not shown). Therefore, we became curious whether the characteristic oncofusion protein EWS-FLI1 is involved in MYC regulation and analyzed its expression upon treatment (Figure 1B and Supplementary Figure S2A). Surprisingly, expression of EWS-FLI1 was reduced after either just JQ1 treatment or combined JQ1 and BEZ235 treatment, which was also confirmed at protein level (Figure 1C). Furthermore, PARP1 and caspase 7 cleavage (Figure 1C) increased after 24 as well as 48 hours JQ1 or combined JQ1 and BEZ235 treatment, indicating induction of apoptosis of ES cells especially after JQ1 and combination treatment.

JQ1 down-regulates an ES specific expression profile

To clarify to which extent JQ1 influences gene expression in ES cells, we carried out microarray analyses on JQ1 treated A673 and TC-71 cells. Analysis of differentially expressed genes using volcano plots indicated 720 and 405 genes significantly up- and down-regulated upon treatment in TC-71 and A673, respectively (P-value < 0.01; Figure 2A). Comparison of expression data between both cell lines at a fold change ± 1.5 by Venn analysis (http://bioinformatics.psb.ugent.be/webtools/Venn/) revealed 811 shared, differentially expressed genes (Figure 2B). Further, a heat map of 244 differentially expressed genes in both cell lines (fold change > 1.8) is shown (Figure 2C). Most of the identified genes were down-regulated after JQ1 treatment (188 genes were down-regulated, 57 were up-regulated, GSE72673). Subsequent gene set enrichment analysis (GSEA) identified a down-regulation of gene sets typical for EWS-FLI1 fusion targets as identified by Zhang and colleagues [31] as well as those for Ewing sarcoma progenitors identified by Riggi *et al.* [32] (Figure 2D), indicating that JQ1 inhibits EWS-FLI1 expression and

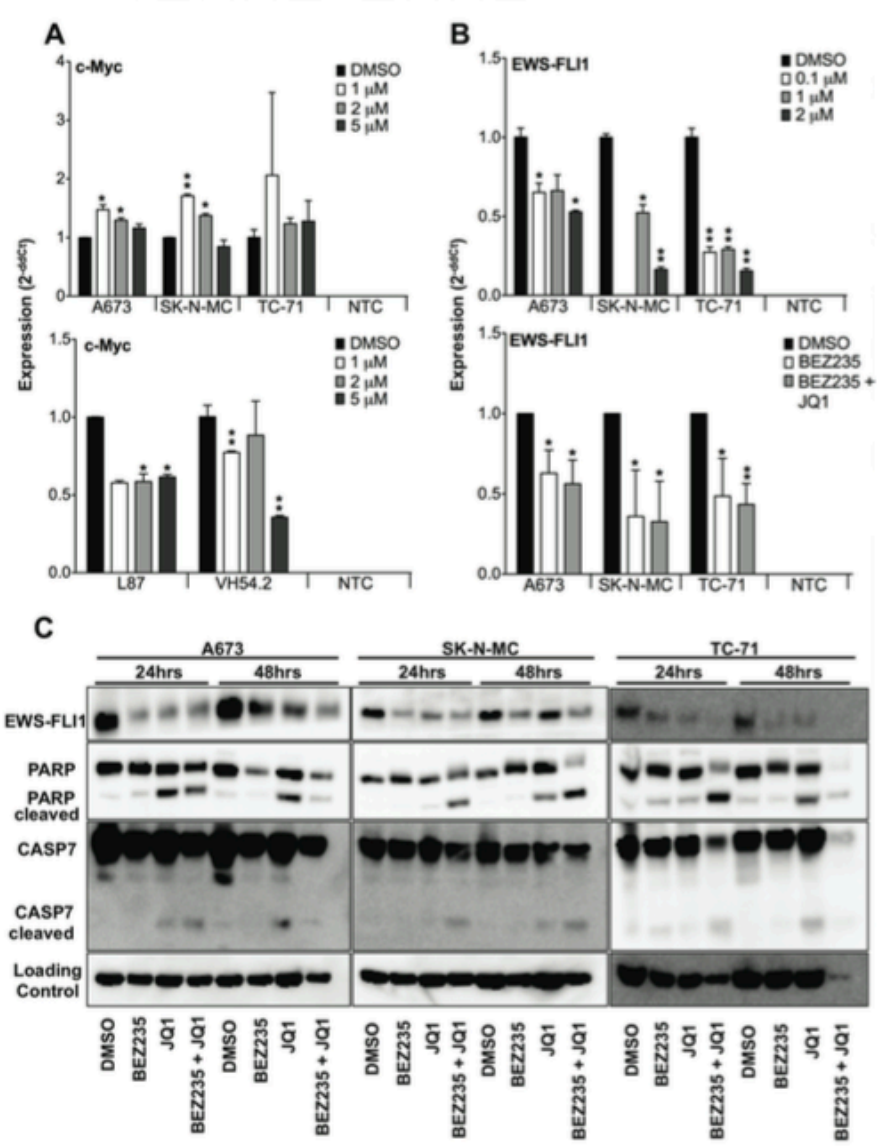

Figure 1: Blockade of BET bromodomain proteins blocks EWS-FLI1 but not MYC expression. A. Top, MYC expression in ES cell lines A673, SK-N-MC and TC-71 and, bottom, in mesenchymal stem cells L87 and VH54.2 after 48hrs JQ1 treatment as measured by qRT-PCR. Data are mean ± SEM; t-test. NTC: non-template control. **B**. Top, different doses of JQ1 inhibit EWS-FLI1 expression in ES cell lines A673, SK-N-MC or TC-71, respectively. Bottom, relative expression of EWS-FLI1 measured by qRT-PCR in A673, SK-N-MC and TC-71 cells after 24hrs treatment with 500nM BEZ235 and 500nM BEZ235 in combination with 2μM JQ1 compared to DMSO control. Data are mean ± SEM; t-test. NTC: non-template control. **C**. Protein level measured by western blot of EWS-FLI1, PARP, CASP7 and loading control. Cells were treated for 24 and 48hrs with 500nM BEZ235, 2μM JQ1, 500nM BEZ235 in combination with 2μM JQ1 compared to DMSO control in A673, SK-N-MC and TC-71 cells. Shown is a representative experiment (n=3). **P*-value < 0.05; ***P*-value < 0.005.

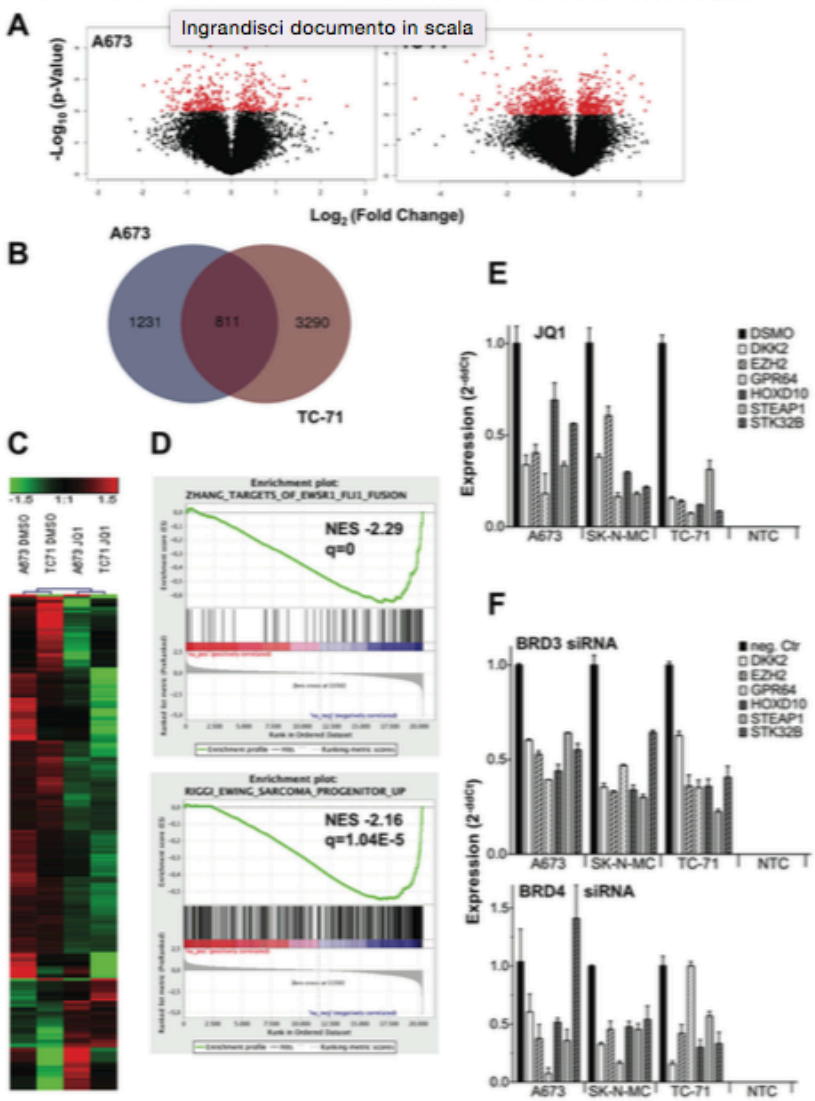

Figure 2: ES expression profile after JQ1 treatment or RNA interference of BRD genes blocks a typical ES associated expression program. A. Volcano plot for DMSO against JQ1 treated ES lines, showing the adjusted significance *P*-value (-$\log_{10}$) plotted over fold change (log2). Red, genes with a significance $P < 0.01$. Microarray data with their normalized fluorescent signal intensities were used (RMA, see *Materials and Methods;* GSE72673). Cells were treated with DMSO or JQ1 for 48hrs, collected, and then analyzed. **B.** Shared genes differentially expressed after JQ1 treatment in 2 different ES lines. For a Venn diagram genes ± 1.5 fold differentially expressed were selected for the analysis (http://bioinformatics.psb.ugent.be/webtools/Venn/). **C.** Heat map of 244 genes, 1.8-fold differentially expressed in 2 different ES lines A673 and TC-71 are shown. Each column represents 1 individual array. **D.** GSEA enrichment plots of down-regulated genes after JQ1 treatment. GSEA: http://www.broadinstitute.org/gsea/index.jsp **E.** Verification of microarray data by qRT-PCR of selected genes. ES specific genes were significantly down-regulated after JQ1 treatment in different ES cell lines. **F.** RNA interference of BRD3 or BRD4, respectively with specific siRNAs affects the same ES specific genes as after JQ1 treatment. Results of qRT-PCRs are shown. Data are mean ± SEM; t-test. NTC: non-template control.

thereby similarly inhibits an ES typical expression profile (Supplementary Table S1). Such genes including *DKK2*, *EZH2*, *GPR64*, *PAPPA*, *STEAP1*, and *STK32B* were shown to be consistently up-regulated and demonstrated to be involved in ES pathogenesis [1, 6, 8, 33, 34]. They were verified to be down-regulated by JQ1 treatment using qRT-PCR, overall confirming results of the microarray analysis (Figure 2E).

RNA interference of different BRDs by specific siRNAs mimics the JQ1 treatment effect in ES

BRD2, *BRD3*, *BRD4* but not *BRDT* genes are well expressed in ES (Supplementary Figure S1). While JQ1 has been reported to be most specific for BRD4 protein, binding to the remaining BRD proteins was also observed, although to a lesser extent [24]. Therefore BRD2, 3 or 4 were transiently down-regulated by specific siRNA in ES and their influence on an ES specific expression profile was analyzed. While BRD2 knock down did not result in any expression changes on such genes (Supplementary Figure S2B), BRD3 and BRD4 knock down uniformly resulted in a similar down-regulation of ES specific genes (Figure 2F) as observed after JQ1 treatment (Figure 2E), concluding that BRD3 as well as BRD4 might be the essential targets of JQ1 treatment hereby repressing the pathognomonic EWS-FLI1 driven expression profile.

JQ1 treatment inhibits proliferation, cell cycle progression and promotes apoptosis

Based on these results, we asked whether the inhibition of the EWS-FLI1 specific expression profile may also affect the growth abilities of ES. Using the xCELLigence assay, we compared contact dependent growth of different ES lines A673, SK-N-MC and TC-71 either treated with 2μM JQ1 or just DMSO (Figure 3A). Neither of the analyzed cells showed a significant increase of cell numbers after JQ1 application. Similarly, contact independent growth of JQ1 treated A673, SK-N-MC and TC-71 revealed a strong reduction of colony formation (Figure 3B) in methylcellulose assay. We subsequently asked whether this reduction of proliferative capacity might be due to changes in cell cycle progression. Flow cytometry analysis for all 3 cell lines depicted a reduction of G2-M phase while an increase in G1 phase was observed after JQ1 treatment in SK-N-MC and TC-71 cells (Figure 3C) as well as an extension of S phase in A673 and SK-N-MC cells. BEZ235 treatment more consistently increased the G1 phase of all 3 cell lines and had no additional effect on the cell cycle when combined with JQ1 (Figure 3C). In addition, the caspase 3 glow assay revealed an increase of apoptosis after JQ1 treatment in A673 and TC-71 that was further increased by combined treatment with BEZ235 in SK-N-MC cells (Figure 3D). These results together with the observed PARP1 and caspase 7 cleavage (Figure 1C) indicate a strong effect of JQ1 on apoptosis induction in ES.

JQ1 reduces tumor growth *in vivo* in a dose dependent manner

As already demonstrated by others [24-27], JQ1 may also have a therapeutic effect *in vivo*. Therefore we also evaluated the therapeutic suitability of ES cells to JQ1 treatment in a xenograft mouse model of $Rag2^{-/-}\gamma c^{-/-}$ mice by implanting tumor cells s.c. into mice. Starting with 50 mg/kg body weight every other day by intraperitoneal injection of JQ1 or vehicle we observed no growth rate reduction on A673 or TC-71 cells, respectively (Figure 4A). Assuming that this concentration of JQ1 might be too low for a pharmaceutically effective supply over two days we also tested more frequent doses on TC-71 cells. Administration of 50 mg/kg twice daily for a period of 14 days resulted in an elongated survival of treated mice (Figure 4B, Top and 4D). To confirm these results we chose SK-N-MC due to its strict dependency on EWS-FLI1 expression. Mice were treated twice daily with 50 mg/kg for 23 days revealing a significant growth reduction (Figure 4B, bottom) and decreased tumor weight (Figure 4E). Further, tumors prepared at the end of the experiment and analyzed immunohistochemically for caspase 3 expression revealed an increased apoptosis rate in tumors treated with JQ1 (Figure 4C). However, further increase to 75 mg/kg twice daily was too toxic and resulted in severe weight loss and death of some mice (data not shown). Overall, both experiments demonstrated a significant inhibition of ES growth *in vivo* at higher JQ1 dosage and the potential therapeutic value of BET bromodomain inhibitors for the treatment of ES.

DISCUSSION

In Ewing sarcoma (ES) the deregulation of components of the epigenetic machinery was previously demonstrated as an important step for tumor formation [1, 8]. Furthermore, it was recently shown that EWS-FLI1 employs divergent chromatin remodeling mechanisms to activate or repress transcription [20, 21]. Herein altered epigenetic marks were identified that generated specific acetyl-lysine moieties on histones and may be targeted by epigenetic reader proteins such as BET proteins (BRD2, BRD3, BRD4). Interestingly, all three BET proteins are well expressed in ES and may be targeted by specific inhibitors of BET proteins such as I-BET151 [23] or JQ1 [24]. Such treatment resulted in dislodgment of BRDs from chromatin in other tumors and inhibition of transcription at key genes involved in apoptosis, cell cycle regulation and oncogenesis [26, 27], [35] [36]. It was demonstrated that JQ1 could block tumor growth and

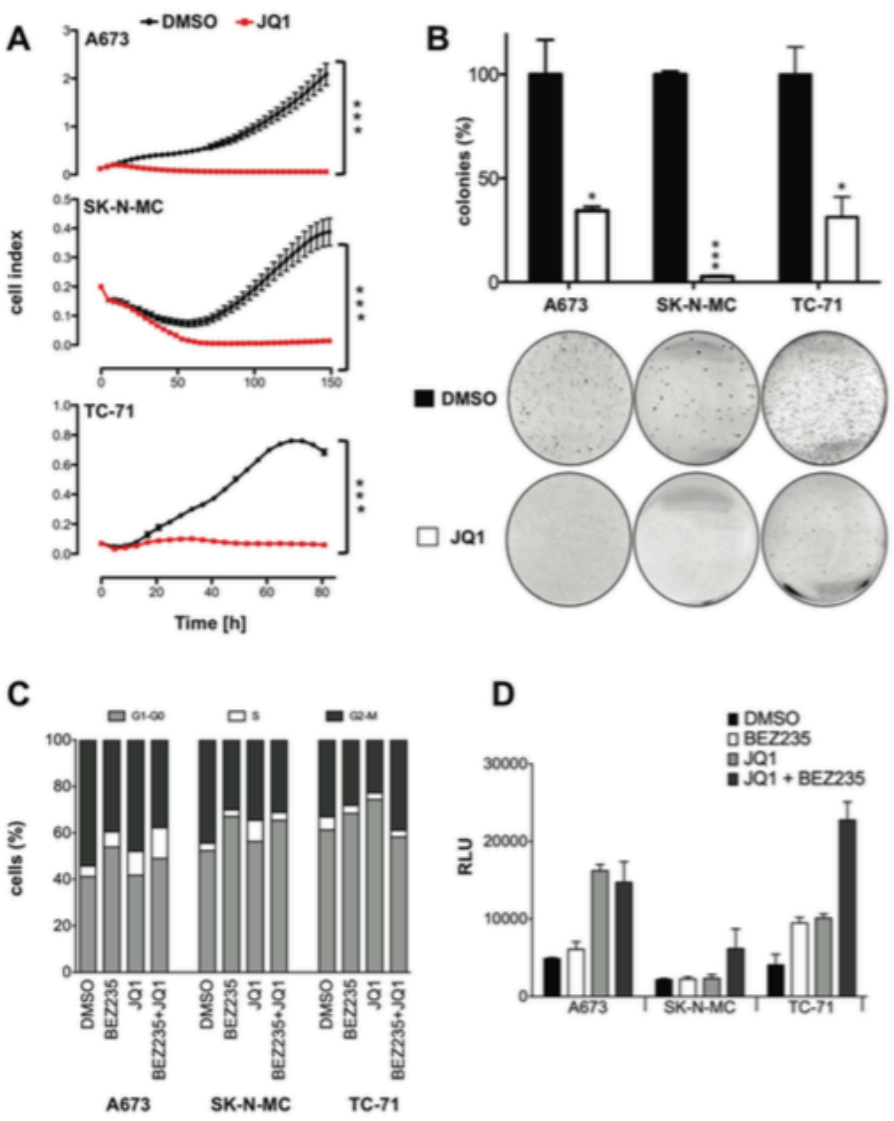

Figure 3: Treatment with JQ1 blocks proliferation, cell cycle progression and induces caspase dependent apoptosis. **A.** Analysis of proliferation of JQ1 treated ES cell lines in comparison to vehicle with xCELLigence. Cellular impedance was measured every 4hrs (relative cell index). Data are mean ± SEM (hexaplicates/group); t-test. **B.** Analysis of anchorage-independent colony formation in methylcellulose of ES lines after JQ1 treatment. Top, data are mean ± SEM of 3 independent experiments (duplicates/group); t-test. Bottom, macrographs show a representative experiment with A673, SK-N-MC and TC-71. **C.** Cell cycle progression upon JQ1 and BEZ235 treatment. Cell cycle analysis after 24 and 48hrs treatment with 500nM BEZ235, 2μM JQ1, 500nM BEZ235 in combination with 2μM JQ1 compared to DMSO treated control in A673, SK-N-MC and TC-71 cells. Shown is a representative experiment (n=3). **D.** Caspase 3/7 activity measured after 24hrs treatment with 500nM BEZ235, 2μM JQ1, 500nM BEZ235 in combination with 2μM JQ1 in A673, SK-N-MC and TC-71. Bars represent mean values expressed as relative light unit (RLU) in percentage of DMSO treated control of 6 biological replicate analyzed in three technical replicates each (n=3; SE<0.01). ***P-value < 0.0005.

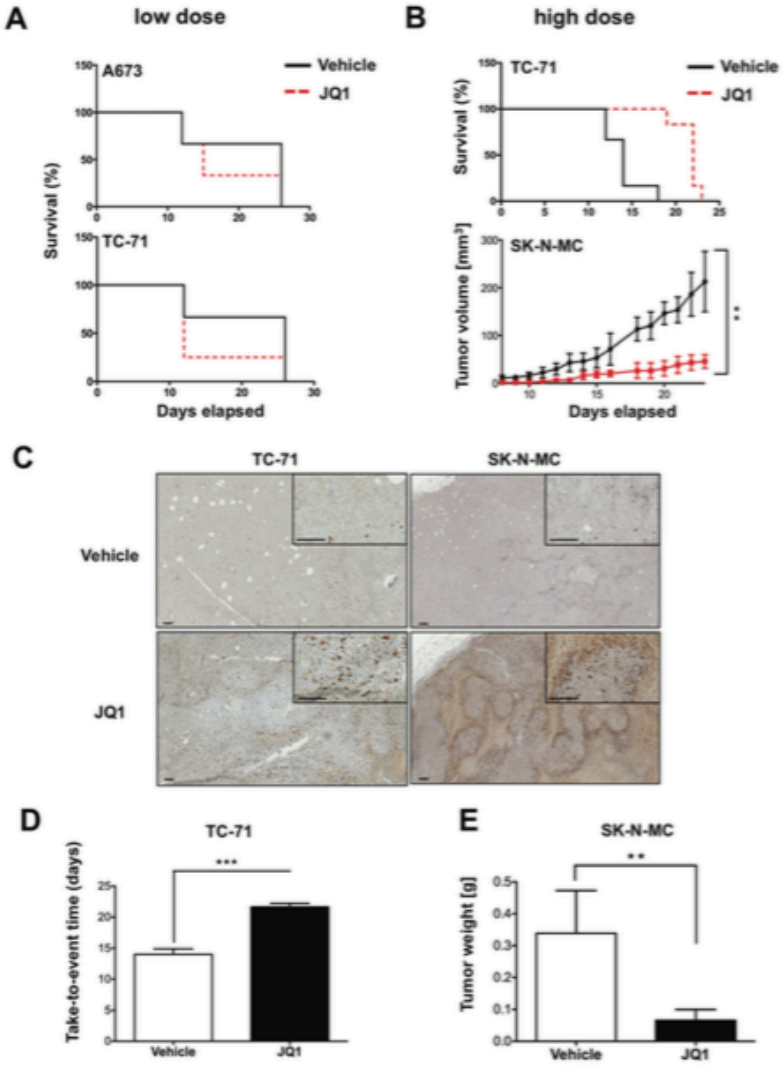

Figure 4: Treatment with JQ1 inhibits ES tumor growth *in vivo*. Evaluation of the therapeutic potential of JQ1 application. Immune deficient Rag2$^{-/-}\gamma c^{-/-}$ mice were injected s.c. with $2x10^6$ ES cells. 5-7 days later these mice received different doses of JQ1 or vehicle i.p., respectively. Delay or inhibition of tumor growth was evaluated. **A.** Mice were either injected with A673 or SK-N-MC cells and 7 days later received 50 mg/kg JQ1 or vehicle every other day. Mice with an average tumor size >10 mm in diameter were considered as positive and killed. Kaplan–Meier plots of individual experiments with 5 mice per group are shown. **B.** Mice were injected with tumor cells s.c. and 5 days later received twice daily doses for 14 to 23 days for TC-71 and SK-N-MC, respectively. Top, survival of TC-71 inoculated mice. Bottom, tumor growth after inoculation with SK-N-MC cells. (6 mice/group). **C.** To analyze intratumoral changes after high dose JQ1 application tumors were collected upon tumor burden (TC-71) or after 23 days (SK-N-MC). The pictures show clear increased expression of cleaved caspase 3 in tumors treated with JQ1. Bar indicates 0.1 mm. **D.** Variation of tumor growth characteristics analyzed as a function of time until tumors reached >1 cm^3 size for TC-71 inoculated mice. **E.** Determined tumor weight of SK-N-MC inoculated mice at the end of the experiment. ***P*-value < 0.005, ****P*-value < 0.0005.

a recurring feature of the consequences of JQ1 / I-BET151 treatment was inhibition of MYC, N-MYC or FOSL [37] expression, respectively in these tumors.

MYC over-expression is well known in ES. Its level of expression seems to be directly regulated via EWS-FLI1 [38]. By use of JQ1 in different ES cell lines we significantly blocked their proliferation and strikingly observed a strong down-regulation of the pathognomonic EWS-FLI1 protein. We saw no inhibition of MYC expression in ES lines whereas its inhibition was readily observed in MSC.

JQ1 treatment suppressed a number of genes typical for ES specific expression profiles [5, 8, 32]. For example GPR64, a new excellent marker of ES [33], was down-regulated after JQ1 treatment. Similarly, the expression of DKK2, a key player of ES invasiveness and osteolytic tumor growth [6], was greatly reduced by JQ1. Further, other genes consistently up-regulated and/or shown to be involved in ES pathogenesis such as *EZH2*, *PAPPA*, *STEAP1*, and *STK32B* [1, 8, 34] were uniformly inhibited by JQ1.

Consistently fewer genes were found to be up-regulated after JQ1 treatment and included genes involved mainly in pathways for cell maturation, differentiation, etc. (GSE72673), confirming that EWS-FLI1 itself is abrogating differentiation programs and is the driver of the immature phenotype of this disease [1, 29].

Interestingly, the JQ1 treatment effect on this expression program of ES lines was mimicked by specific siRNA-mediated knock down of BRD3 or BRD4 but not BRD2 expression, demonstrating that not only BRD4 is an important epigenetic reader protein in ES. Displacement of BRD3/4 by specific inhibitors has already been shown in MLL-fusion positive leukemia [23]. While BRD3 seems to preferentially associate with hyper-acetylated chromatin along the entire lengths of transcribed genes [39], BRD4-binding has also been observed in enhancer regions [40]. To what areas of chromatin BET proteins bind in ES has to be further investigated.

JQ1 treatment not only suppressed an ES specific expression profile but also blocked contact dependent and independent proliferation of different ES lines. This seems to be due to a partial G1 arrest and S phase elongation of the cell cycle as demonstrated previously [24]. In addition, induction of apoptosis as demonstrated by PARP1-, CASP7-cleavage and increased CASP3 activity seems to significantly contribute to the reduction of the proliferative ability of ES lines. Single or combination treatment with the PI3K/mTOR inhibitor BEZ235 [41] did increase apoptosis of ES cell lines although single treatment with BEZ235 was less effective than JQ1 application.

However, a number of substances initially also demonstrated efficacy in preclinical models such as single treatment with ARA-C [42] or anti-IGFR [43] but in phase I clinical trials delivered transient [29] or disappointing results [44], emphasizing the need to combine drugs that based on the biology of this tumor may result in synergistic growth inhibitory effects.

For example, treatment with BEZ235 clearly inhibited EWS-FLI1 expression and in combination with JQ1 further increased apoptosis induction indicating that combination treatment of JQ1 with PI3K/mTOR inhibition should be a promising strategy for future therapy of ES.

Also, combination treatment of JQ1 with substances like YK-4-279 that directly binds to EWS-FLI1 and inhibits its oncogenic activity [45, 46] via blockade of specific protein interaction with factors important for mRNA splicing [47] and transcription [45] may result in synergistic effects on tumor growth and needs to be explored in preclinical models of ES.

Similarly, combination treatment with EZH2 inhibitors such as GSK126 [48, 49] may further increase therapeutic efficacy and due to potential synergistic effects decrease JQ1 dose levels required for successful treatment of ES.

Here, we at first demonstrated that tumor development was dose dependently suppressed by intra-peritoneal JQ1 application in a xeno-transplant model of ES–bearing immune deficient $Rag2^{-/-}\gamma c^{-/-}$ mice. Therapeutic efficacious doses, although high, were within the range published previously [24, 27, 35]. Overall, our results demonstrate that MYC or EWS-FLI1 mediated pathognomonic expression programs may be similarly targeted by BET bromodomain inhibition, casting BET protein inhibition appropriate as a potential platform for future combination therapy of this disease.

MATERIALS AND METHODS

Cell lines

ES lines (SK-N-MC and TC-71), neuroblastoma lines (CHP126, MHH-NB11, SHSY5Y and SIMA) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). A673 was purchased from ATCC (LGC Standards, Teddington, UK). Mesenchymal stem cell lines L87 an V54.2 were described previously [33]. Cells were maintained in a humidified incubator at 37 °C in 5-8 % CO_2 atmosphere in RPMI 1640 (Life Technologies, Carlsbad, CA, USA) containing 10 % heat-inactivated fetal bovine serum (Biochrom, Berlin, Germany) and 100 µg/ml gentamicin (Life Technologies). Cell lines were checked routinely for purity (e.g. EWS-FLI1 translocation product, surface antigen or HLA-phenotype) and Mycoplasma contamination.

RNA interference (RNAi)

For transient RNA interference cells were transfected with small interfering RNA (siRNA) as described previously [1]. To test transfection efficiency and gene silencing RNA was extracted and gene expression assessed by quantitative Real Time-PCR. siRNA sequences are provided in the Supplementary Information.

Quantitative real time-PCR (qRT-PCR)

Total RNA was isolated and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific AG) according to the manufacturer's instructions. Differential gene expression was then analyzed by qRT-PCR using TaqMan Universal PCR Master Mix and fluorescence detection with Step One Plus Real-Time PCR or ABI 7900 instrument (Thermo Fisher Scientific AG) as described previously [1, 33]. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All experiments were performed at least in duplicate for each cell line. A list of used assays is provided in the Supplementary Data. NTC: non template control.

Proliferation assay

Cell proliferation was measured with an impedance-based instrument system (xCELLigence, Roche/ACEA Biosciences, Basel, Switzerland) enabling label-free real time cell analysis. Briefly, 4 - 10 x 10^3 cells were seeded into 96-wells with 200 µl media containing 10 % FBS and allowed to grow up to 150 hours. Cellular impedance was measured periodically every 4 hours and 2µM JQ1 or DMSO was added.

Colony forming assay

Cells were seeded in duplicate into a 35 mm plate at a density of 5 x 10^3 cells per 1.5 ml methylcellulose-based media (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and cultured for 10-14 days at 37 °C / 5 % CO_2 in a humidified atmosphere. 2µM JQ1 or DMSO was added

Immunoblotting

5 x 10^5 A673, SK-N-MC or TC-71 cells were treated with 500nM BEZ235, 2µM JQ1, 1µM JQ1, 500nM BEZ235 in combination with 2µM JQ1 or DMSO as controls, washed twice with PBS and harvested in lysis buffer containing 50mM NaH_2PO_4 (pH 7.5), 150mM NaCl, 1% Triton X-100, 1mM Na3OV4, 5mM Na-pyrophosphate, 40nM NaF, 1mM EGTA supplemented with protease inhibitor cocktail (Complete + 1mM EDTA, Roche Diagnostics AG). Protein concentration was determined by BCA (Thermo Fisher Scientific AG). 10–30µg of protein extract was resolved on 4-12% SDS-PAGE and transferred onto nitrocellulose membrane (Thermo Fisher Scientific AG). Primary antibodies were used as follows: anti-FLI1 monoclonal antibody (MyBioSource LLC, San Diego, USA), anti-PARP rabbit polyclonal antibody (Cell Signaling Technology, Danvers, USA), anti-Caspase7 antibody (Cell Signaling) and loading control (anti-β-tubulin (Sigma-Aldrich, St. Louis, USA) or GAPDH (Cell Signaling)). After incubation with the appropriate secondary peroxidase-conjugated antibodies, detection was performed with the ECL chemiluminescence reagent (Amersham Biosciences, Little Chalfont, UK).

FACS analysis

Treated cells were washed with PBS, collected, fixed with 70% Ethanol for 2hrs on ice and stained with PI solution- for 1hr at room temperature- 20 µg/ml PI (Sigma-Aldrich), PBS, 0.1% TritonX, 200 µg/ml RNAse A for measurement with a FACS Canto. Data were analyzed using Flow Jo program (Flow Jo LLC., Ashland, OR, USA).

Casp3/7 assay

4 x 10^3 A673, SK-N-MC or TC-71 cells, were plated in a 384-well plate previously coated with 0.2% gelatin. After 24hrs, cells were treated with 500nM BEZ235, 2µM JQ1, 500nM BEZ235 in combination with 2µM JQ1 or DMSO as controls. 24hrs after treatment Caspase 3/7 reagent (Promega AG) was added in each well and luminescence was measured.

Microarray analysis

Biotinylated target cRNA was prepared as previously described [1]. A detailed protocol is available at www.affymetrix.com. Samples were hybridized to Affymetrix Human Gene 1.0 ST microarrays and analyzed by Affymetrix software expression console, version 1.1. For the data analysis, robust multichip average (RMA) normalization was performed, including background correlation, quantile normalization, and median polish summary method. Probes of the normal body map (NBA) included tissues of normal PBMC, bone marrow, spleen, thymus, stomach (2), small intestine, colon w/ mucosa, heart, liver, lung, skeletal muscle, brain (whole), brain cerebellum, spinal cord, trachea, salivary gland, prostate,

testis, uterus, fetal brain, and fetal liver. Array data were submitted at GEO (GSE45544).

Animal model

Immune deficient Rag2$^{-/-}\gamma c^{-/-}$ mice on a BALB/c background were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and maintained in our animal facility under pathogen-free conditions in accordance with the institutional guidelines and approval by local authorities (Regierung von Oberbayern). Experiments were performed in 6-20 week old mice.

In vivo experiments

To examine *in vivo* tumorigenicity, 2-3 x 10^6 ES cells were injected subcutaneously into the inguinal region of immune deficient Rag2$^{-/-}\gamma c^{-/-}$ mice. JQ1 was handled and dissolved as recommended by the Bradner lab and administered at 50 mg/kg body weight intra peritoneal either twice daily or every other day. Mice were monitored daily and tumor xenografts were measured with digital calipers, and tumor volume was calculated as (L x W^2) / 2, where L is length and W is width. Experimental endpoints were determined by completion of treatment or attainment of tumor burden exceeding 1 cm^3. Upon reaching endpoints, mice were humanely euthanized and tumors excised and characterized.

Histology

Histological analysis of tumor specimens was performed in a minimum of 5 mice per group. Tissues organs were fixed in phosphate buffered 4% formaldehyde and paraffin embedded. 3-5µm thick sections from all tissues were stained with hematoxylin and eosin (H&E). Apoptosis was evaluated by immunohistochemistry (IHC) using cleaved Caspase 3 (Cell Signaling) as primary antibody. The IHC was performed using the streptavidin–peroxidase method with an automated immunostainer (DiscoveryXT; Roche, Penzberg, Germany), All sections were reviewed and interpreted by two pathologists.

Statistical analyses

Data are mean ± SEM as indicated. Differences were analyzed by unpaired two-tailed student's t-test as indicated using Excel (Microsoft, Redmond, WA, USA) or Prism 5 (GraphPad Software, San Diego, CA, USA); P-values < 0.05 were considered statistically significant (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$). Volcano plots were drawn using R, a free software environment available at http://www.r-project.org/.

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CONFLICTS OF INTEREST

The authors disclose no potential conflicts of interest.

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Authors' contribution

TH, CG, OS, J.C-W. and GHSR performed experiments. TH, CG, BS, FN and GHSR analyzed data. BS, FKN, SB and GHSR initiated the project. TH and GHSR wrote the paper.

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Targeting the EWS-ETS transcriptional program by BET bromodomain inhibition in Ewing sarcoma

Supplementary Material

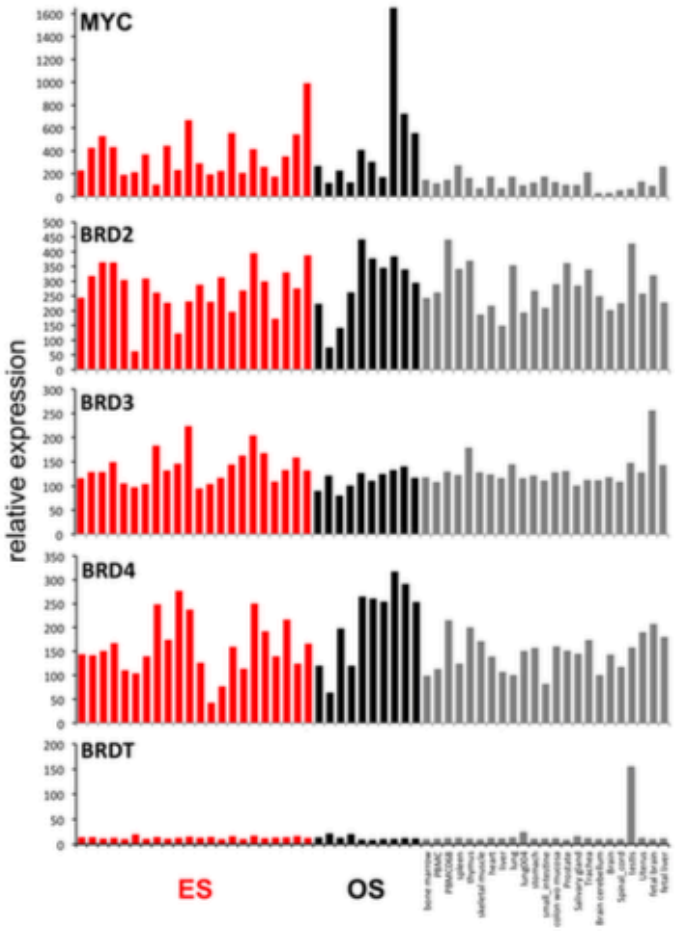
Methods

Small interfering RNAs used:

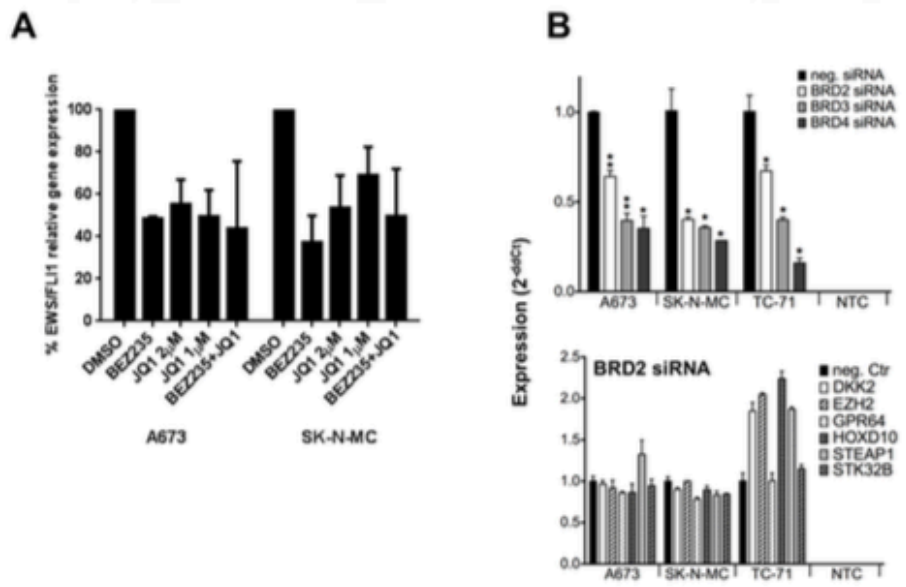
All siRNAs were purchased from Qiagen. Sequences are as follows: siBRD2_8 5'-GUAGCAGUGUCACGCCUUATT-3' (sense) and 5'-UAAGGCGUGACACUGCUACTT-3' (antisense); siBRD3_8 5'-GCCGCCUGUCGUCAAGAAATT-3' (sense) and 5'-UUUCUUGACGACAGGCGGCGT-3' (antisense); siBRD4_9 5'-GGACUAGAAACUUCCCAAATT-3' (sense) and 5'-UUUGGGAAGUUUCUAGUCCAT-3' (antisense) and control non silencing siRNA 5'-UUCUCCGAACGUGUCACGU-3' (sense) and 5'-ACGUGACACGUUCGGAGAA-3' (antisense).

Primers and assays used for RT-PCR

For EWS-FLI1 detection, the following primers 5'-TAGTTACCCACCCCAAACTGGAT-3' (sense) 5'-GGGCCGTTGCTCTGTATTCTTAC-3' (antisense) and probe 5'-FAM CAGCTACGGGCAGCAGAACCCTTCTT-TAMRA-3' were designed. Inventoried TaqMan Gene Expression Assays (Thermo Fisher Scientific AG) were used for the genes *DKK2* (Hs00205294_m1), *GAPDH* (Hs99999905_m1), EZH2 (Hs01016789_m1), BRD2 (Hs01121986_g1), BRD3 (Hs00201284_m1), BRD4 (Hs04188087_m1), STEAP1 (Hs00185180_m1), GPR64 (Hs00971379_m1), HOXD10 (Hs00157974_m1), STK32B (Hs00179683_m1), c-Myc (Hs00153408_m1) .



Supplementary Figure S1. Microarray analysis of primary Ewing and Osteosarcoma. Tumor RNA was hybridized onto Human Gene 1.0 ST microarrays (Affymetrix; Santa Clara, CA), analyzed by Affymetrix software expression console, version 1.1. and compared to normal tissue (GSE45544, GSE73166). Signal intensities in Ewing and osteosarcoma in comparison to normal tissue for MYC and BET genes (BRD2, BRD3, BRD4, BRDT) are shown.



Supplementary Figure S2. JQ1 and BEZ235 treatment reduces EWS-FLI1 expression *in vitro*. A. Relative expression of EWS-FLI1 measured by qRT-PCR after 24hrs treatment with 500nM BEZ235, 2µM JQ1, 1µM JQ1 and 500nM BEZ235 in combination with 2µM JQ1 compared to DMSO control in SK-N-MC and A673 cells. Shown are representative experiments (n=3). **B**. Relative Expression of BRD2, 3 or 4 after 24 - 66hrs of RNA interference determined by qRT-PCR and analysis of JQ1 regulated genes after BRD2 knock down.

Supplementary Table S1*

Gene Symbol	Gene Description	Q-JQ1
GPR64	G protein-coupled receptor 64	0.243
JMJD1C	jumonji domain containing 1C	0.249
SMARCAD1	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1	0.275
STAG2	stromal antigen 2	0.283
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	0.288
PAPPA	pregnancy-associated plasma protein A, pappalysin 1	0.313
PCDHB5	protocadherin beta 5	0.325
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	0.334
STEAP1	six transmembrane epithelial antigen of the prostate 1	0.367
HDAC9	histone deacetylase 9	0.371
RGS4	regulator of G-protein signaling 4	0.380
CCNA1	cyclin A1	0.382
STEAP2	six transmembrane epithelial antigen of the prostate 2	0.388
HOXB2	homeobox B2	0.393
TET2	tet oncogene family member 2	0.405
IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	0.425
HIST1H2AB	histone cluster 1, H2ab	0.427
LIPI	lipase, member I	0.436
HIST1H1T	histone cluster 1, H1t	0.442
HOXD10	homeobox D10	0.445
HIST1H2BB	histone cluster 1, H2bb	0.448
CCNB2	cyclin B2	0.451
JARID2	jumonji, AT rich interactive domain 2	0.458
HDAC8	histone deacetylase 8	0.470
TET1	tet oncogene 1	0.473
TET2	tet oncogene family member 2	0.477
STK32B	serine/threonine kinase 32B	0.480
DKK2	dickkopf homolog 2 (Xenopus laevis)	0.480
SMARCAL1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a-like 1	0.482
CCNA2	cyclin A2	0.483
JHDM1D	jumonji C domain containing histone demethylase 1 homolog D (S. cerevisiae)	0.492
EZH2	enhancer of zeste homolog 2 (Drosophila)	0.495
PAX7	paired box 7	0.499

*Selected genes down-regulated after JQ1 inhibition in TC-71 cells are shown.

5.3 Phosphorylation of EWS-FLI1 modulates growth of Ewing sarcoma cells

Ready for submission

Contribution: LL established cell lines and started the project. I performed EMSA, taqman and western blot upon doxycycline treatment of HEK and A673 shEWS/FLI1 cells. I performed in vivo experiment (Fig. 4/ Fig. 5/ Suppl. Fig. 3).
LL wrote the manuscript.

Phosphorylation of EWS-FLI1 modulates growth of Ewing sarcoma cells

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Abstract

Ewing sarcoma (ES) is a highly aggressive pediatric tumor that affects bones and soft tissues, which is characterized by a balanced chromosomal translocation that leads to the expression of the oncogenic transcription factor EWS-FLI1 unique to ES tumors. Continued expression of EWS-FLI1 is crucial for tumor cell survival, and therefore it represents an attractive therapeutic target. Here, we hypothesized that post translational modifications such as phosphorylation would offer a unique opportunity to indirectly modulate the activity of the fusion protein. Hence, we identified novel amino acid residues being phosphorylated in EWS-FLI1 by mass spectrometry directly from ES cells. Using EWS-FLI1 target genes as read-out for fusion protein activity in heterologous cells, we characterized S287 phosphorylation as being important for full transcriptional activity. Using novel stable cell line systems that allow overexpression of EWS-FLI1 mutants while downregulating the endogenous protein, we demonstrated a lower proliferation rate of ES cells both in vitro as well as in vivo. Mechanistically, a phospho-deficient S287A mutant showed diminished DNA binding activity at a conserved EWS-FLI1 binding motif.

Hence, we conclude from these experiments that phosphorylation of EWS-FLI1 is necessary for full activity of the fusion protein, and further characterization of upstream kinases might help to discovery of new target options for the treatment of Ewing sarcoma.

Introduction

Variations in the extracellular environment cause changes in gene expression that lead to appropriate physiological responses, mostly driven by transcription factors (TFs). Integration of multiple intracellular signalling pathways is essential to control the function of TFs, mostly via post translational modifications to regulate cellular localization, protein-protein interactions, protein-DNA interactions and stability.

One of the most common and tightly regulated mechanism for signal transduction is phosphorylation and dephosphorylation of proteins, including TFs [1]. In the case of diseases caused by dysregulation of gene expression such as some cancer types, targeted inhibition of the causative TF would be the best therapeutic option. Since direct pharmacological inhibition of TFs remains challenging, elucidating signalling pathways that are responsible for regulating the phosphorylation state of a TF is a field of special interest because they represent potential targets for therapeutic intervention.

Ewing sarcoma (ES) is a highly aggressive pediatric tumor that affects bone and soft tissues. ES is a classic example of a malignancy driven by an oncogene generated from a unique chromosomal translocation that gives rise to a specific fusion gene, in 85 % of all ES cases this is the t(11;22)(q24;12) translocation leading to the expression of the aberrant TF called EWS-FLI1 [2-4]. ES cells are dependent on continuous activity of EWS-FLI1 [5] [6] [7] and its unique presence in tumor cells only makes EWS-FLI1 an ideal target for treatment. This is reinforced by the fact that pediatric tumors, in contrast to adult tumors, harbor very low numbers of somatic mutations, rendering them more challenging for development of targeted therapies [8]. Indeed, the fusion protein is the sole genetic abnormality in a large number of patients and therefore there have been no alternative therapeutic strategies to the conventional treatments (chemotherapy, radiotherapy and surgery) introduced until now in upfront therapy. Furthermore, ES patients frequently develop resistance towards the current treatment modalities and patients in such cases have a poor prognosis with 5-year survival rate of no more than 50% [9].

The oncogenic activity of EWS-FLI1 is mostly mediated through inappropriate regulation of target genes. Therefore, most of the investigations that focused on these downstream targets revealed important candidates implicated in cellular transformation such as NKX2.2 [10], IGF1 [11], TGFBRII [12], IGFBP3 [13] and LOX [14] among others. In contrast, less advances have been made in understanding the molecular regulation of EWS-FLI1 itself. Interestingly, EWS-FLI1 and also EWSR1 have been demonstrated to be post translationally modified by additions of N-acetylglucosamine [15], acetylation [16], and phosphorylation [17] [18]. However, not much is known about the importance of these modifications for transcription factor function, which consequently might also affect tumor aggressiveness and prognosis, and therefore play a role in therapy resistance and relapse. The fusion protein might in addition be regulated on other levels such as transcriptional regulation of its expression [19] and protein-protein interactions [20]. These data all suggest that upstream signalling pathways

responsible for post translational modifications play an important role in EWS/FLI1 activity and biology.

Therefore, we have analyzed here the post translational modifications present in EWS-FLI1 purified directly from Ewing cell lines by mass spectrometry. Focusing on EWS-FLI1 phosphorylation we have identified a novel phosphorylation site S287 which is important for DNA binding. Mutation of this site decreases DNA binding, inhibits growth of Ewing cell lines and diminishes its clonogenic capacity. Furthermore, in a xenograft mouse model ES cells harbouring a S287A mutant, tumor growth is reduced. Hence, pharmacological inhibition of the protein kinase(s) responsible for phosphorylation of S287 of EWS-FLI1 may hold promise as interesting addition to current chemotherapeutic treatment modalities of ES.

Materials and Methods

Cell lines

Ewing sarcoma cell lines A673, SKNMC, RD ES and TC71 were cultured in RPMI supplemented with 10% FBS (Sigma Aldrich), 2 mM glutamine (BioConcept, Allschwil, Switzerland) and 100 U/ml penicillin/streptomycin (ThermoFisher Scientific AG, Reinach, Switzerland) at 37°C in 5% CO2. Additionally, dishes were pre-coated with 0.2% gelatine (Sigma Aldrich). HEK293T cells and RD (Rhabdomyosarcoma) cells were cultured in DMEM (Sigma Aldrich, Buchs, Switzerland) supplemented as described above.

Plasmids and cloning

The coding sequence for human EWS-FLI1 and FLI1 were subcloned into the Not1 site of pCMV-3xflag vector (Sigma Aldrich). Plasmids used for Global Protein Stability were previously described (Gierisch et al. under revision). The pRSIT-U6Tet-shFLI1.t5322-hPGK-TetRep-2A-TagGFP2-2A-Puro vector with shRNA against EWS/FLI1 was purchased from Cellecta Inc. with the following sequence targeting the 3´UTR of FLI1: shFLI1_t5322: 5'CGTCATGTTCTGGTTTGAGAT 3´. For retroviral expression the coding sequence of EWS/FLI1 was cloned in the pMSCVpuroFlag_cMycT58A plasmid received from AddGene (Plasmid #20076). cMycT58A was excised and EWS/FLI1 sequence inserted between XhoI and EcoRI sites.

For lentiviral expression, pRR-CMV-Bleo-EF1-MCS was purchased from Cellecta and EWS/FLI1 coding sequence was cloned in Nhe1 site. Cloning was performed by In-Fusion cloning HD (Clontech Laboratories Inc., Mountain View, CA, USA) according to

manufacturer's protocol. All mutations were introduced using site-directed mutagenesis. All clonings were verified by sequencing.

Transient transfection

For HEK293T cells, DMEM complete medium was mixed with PolyethylenimineMax (Polyscience,Cham, Switzerland) and plasmid for 15min and added to the cells for 48h. For A673 cells JetPrime (Polyplus Transfections, Illkirch, France) reagent was used according to manufacturer's instruction in antibiotics-free RPMI medium.

Viral production and transduction

For shRNA lentivirus production, the third generation lentiviral expression system was used where packaging plasmids REV and MDL and envelop plasmid VSV were transfected together with the shRNA expression plasmid using JetPrimeTM into 1X 175cm² flask containing $5{\cdot}10^6$ HEK293T cells. Medium was replaced 24hrs after transfection and supernatant containing virus was harvested after additional 48hrs. Viral supernatant was cleared by centrifugation, filtered and aliquots were frozen and used only once when required. For 3X Flag-EWS/FLI1 lentivirus production, 2X 175cm² flasks containing $5{\cdot}10^6$ HEK293T cells each were transfected with the corresponding plasmids. Medium was replaced 24hrs after transfection and supernatant containing virus was harvested after additional 48hrs, cleared by centrifugation, filtered and concentrated (Amicon® Ultra 15 mL, Millipore) to 3 ml. Aliquots of 1 ml were frozen and used only once when required.

A673 and SKNMC cells were stably transduced with lentiviral particles supplemented with 10µg/ml polybrene (Sigma Aldrich). The day before transduction, cells were plated in 12 well plates to a density of 50000 cells / well. Transduction was performed by centrifugation at 800 G for 1h30min at 32°C. Cells were selected during 10 days with 1 mg/ml puromycin. After selection, 0.1 µg/ml doxycycline was added in the medium to induce expression of the shRNA. Knock down of EWS/FLI1 was confirmed by western blotting. A673-shRNA and SKNMC-shRNA cell lines were additionally transduced with lentivirus containing the wild type and mutants 3X Flag-EWS/FLI1 expression plasmids. Transduction was performed as described above using 500 µl of concentrated virus supernatant. Selection with 2 mg/ml bleomycin began 9 days post-transduction and was maintained for 10 days until all non-transduced control cells detached. Afterwards, cells were maintained under selection pressure by 0.5 mg/ml puromycin. RD cells were transduced with retroviral particles containing the wild type and mutant 3X Flag-EWS/FLI1 expression plasmids as described above. All cells

were cultivated at 37°C in 5 % CO_2. Generation of the A673 shRNA and SKNMC shRNA cell lines expressing wt or mutants of EWS/FLI1 were confirmed by sequencing of genomic DNA.

Immunoblotting

Cells were washed with PBS and harvested in complete lysis buffer containing 50mM Tris/HCl, 150mM NaCl, 50mM NaF, 5mM Na4P2O7, 1mM Na3VO4 and 10mM ß-glycerolphosphate, 1% TritonX with protease inhibitor cocktail (complete with 1 mM EDTA, Roche). Protein concentration was determined by BCA (Thermo Fisher Scientific AG). Total cell extracts were separated using 4-12% BisTris NuPAGE pre-cast gels (ThermoFisher Scientific AG) and transferred to nitrocellulose membranes (GE Healthcare). After blocking with 5% milk powder in PBS/0.1% Tween, the membrane was incubated overnight with the primary antibody followed by 1h of HRP-linked secondary antibody at room temperature. Proteins were detected by chemiluminescence using Amersham ECL detection reagent (GE Healthcare, Glattbrugg, Switzerland) or SuperSignalTM Western blotting reagent (ThermoFisher Scientific AG). The following commercial antibodies were used: anti-FLI1 (anti rabbit, MyBiosouerce LLC, 1:1000), anti-Flag M2 antibody (anti mouse F1804, Sigma-Aldrich, 1:1000), anti-β-tubulin I mouse mAb (Sigma-Aldrich, 1:40,000), anti-GAPDH (1:1000, Cell Signaling), IgG HRP-linked antibody (anti-mouse 7076; Cell Signaling, Bioconcept, 1:2000), IgG HRP-linked antibody (anti-rabbit 7074, Cell Signaling, Bioconcept, Allschwil, 1:2000).

Immunoprecipitation

For MS analysis, A673 cells transiently expressing 3x Flag-EWS/FLI1 or 3x Flag-FLI1 from three confluent 15 cm dishes were lysed in 1 ml complete lysis buffer and incubated for 30 min at 4°C with Dynabeads® Protein G (Novex by Life Technologies) coupled to monoclonal anti-Flag M2 antibody. Beads were washed three times with lysis buffer, and proteins eluted at room temperature using 3X Flag-peptide (Sigma-Aldrich).

For EWS/FLI1 endogenous immunoprecipitation, A673, SKNMC, TC71 and RD ES cells were lysed from 10 cm dish in 500 µl complete lysis buffer and incubated for 3 hrs at 4°C with Dynabeads® Protein G coated with mouse monoclonal FLI1 antibody (Santa Cruz). Beads were washed three times with lysis buffer, proteins eluted in 1x RotiLoad sample buffer (Carl Roth) at 90°C and analyzed by Western blotting. Phospho-S287 antibody was

developed by Eurogentec with the peptide sequence SGLNKS*PPLGGA (S*=phosphorylated).

Cell cycle analysis

Treated cells were trypsinized (0.05% Trypsin, w/o EDTA, Invitrogen Life Technology), washed in 1X PBS, fixed with 70 % ethanol for 2 hrs on ice and stored at -20°C. Before cell cycle measurement, they were washed in PBS, resuspended in 300 µl PBS containing propidium iodide (20 µg/ml PI (Sigma-Aldrich), 0.1% TritonX, and 200 µg/ml RNAse A and incubated at 4°C in the dark for 15 min before flow cytometry analysis (Beckman Coulter Cytomics FC500; Hialeah, FL). Data were processed with FlowJo software (Treestar, Ashland, OR).

Immunofluorescence

A673 cells were seeded on cover slides for 24hrs and transiently transfected with flag tagged plasmids for additional 48hrs. After fixing with 4% PFA (Carl Roth, Arlesheim, Switzerland), cells were permeabilized and stained with anti-Flag antibody (1:50, Sigma Aldrich) in 4% horse serum (Sigma Aldrich) and 0.1% PBS-TritonX overnight. Fluorescent secondary antibody (1:500, Alexa-488 anti-mouse, Sigma Aldrich) in PBS with 4% horse serum was applied for 1h. Cover slides were fixed on objective glass with DAPI (Vectashield, Vector laboratories Inc., Burlingame, CA, USA) and analyzed with an inverted microscope (Carl Zeiss Microscopy LLC, Thornwood, NY, USA).

Clonogenic assay

A673-shRNA-Flag-EWS/FLI1 cell lines were plated on 0.2% gelatin coated 6 well plate (500 cells/well) and 0.1 µg/ml of doxycycline was added daily for 21 days. Colonies were fixed with paraformaldehyde (PFA), stained with crystal violet (Sigma) and manually counted.

Cell growth

A673-shRNA-Flag-EWS/FLI1 cell lines were plated on 12 well plates (100000 cells/well). From next day 0.1 µg/ml of doxycycline was added daily. Cells from 2 independent wells were trypsinized, collected and counted for each time point. For cell proliferation assay, A673-shRNA-Flag-EWS/FLI1 cell lines were plated on 96 well plate (2000 cells/well) with 0.1 µg/ml of doxycycline added daily. BrdU incorporation was measured by absorbance following the manufacture instructions (Cell proliferation Elisa BrdU colorimetry, Roche).

Quantitative PCR

Total RNA was extracted using Qiagen RNeasy Kit (Qiagen Instruments AG, Hombrechtikon, Switzerland). cDNA synthesis was performed using High-Capacity Reverse Transcription Kit (Applied Biosystems by ThermoFisher Scientific AG). Quantitative PCR was performed under universal cycling conditions on an ABI 7900 instrument using the following commercially available target probes (Applied Biosystems by ThermoFisher Scientific AG) and TaqMan gene expression master mix (Life Technologies, Zug, Switzerland): EWSR1-FLI1: Hs03024807_ft, PHLDA1: Hs00378285_g1, CAV1: Hs00184697_m1, NKX2.2: Hs00159616_m1, NR0B1: Hs03043658_m1, GAPDH: Hs99999905_m1, LOX: Hs00942480_m1, NPY1R (Hs00702150_s1), IGF1 (Hs01547656_m1), SOX2 (Hs04234836_s1), NGFR (Hs00609977_m1), EZH2 (Hs00544833_m1).

Data were analyzed using SDS 2.2 software (Thermo Fisher Scientific AG). Cycle threshold (CT) values were normalized to GAPDH. Relative expression levels were calculated using the ΔΔCT method based on experiments performed in triplicates. Geometric mean values and the 95% confidence interval were calculated with the GraphPad prism software (San Diego, CA, USA) based on three biologic replicates.

Electrophoretic mobility shift assay (EMSA)

$5x10^6$ cells were treated with Doxycline 0.1 mg/ml for 48 hrs, washed once with PBS and lysed in lysis buffer containing 50 mM NaH_2PO_4 (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM Na_3OV_4, 5 mM Napyrophosphate, 40 nM NaF, and 1 mM EGTA supplemented with protease inhibitor cocktail (Complete + 1 mM EDTA, Roche Diagnostics AG).

40 µg of total extract, 100 nM of biotinylated oligo, 4 µM of unlabelled probe, 1 µl of anti-FLI1 (Myobiosource) or anti Actin antibodies (Cell Signalling) were mixed.

Oligonucleotides were ordered from Microsynth AG, Balgach, Switzerland) and annealed with Annealing Buffer (10 mM Tris, 1 mM EDTA, 50 mM NaCl pH 8.0):

FLI1_Forward: ATG TAG ACC GGA AGT AAC (biotin);

FLI1_Forward: ATG TAG ACC GGA AGT AAC TA;

FLI1_Reverse: TAG TTA CTT CCG GTC TAC AT;

Xenograft studies

A673-shRNA-Flag-EWS/FLI1 cells were trypsinized into single-cell suspensions. After washing once with PBS, $5x10^6$ cells were diluted in 100 µl of PBS and engrafted

subcutaneously in 8-12 weeks-old NOD/Scid il2rg-/- mice (male and female, 20–25 g; Charles River). When palpable tumors were detected, doxycyclin (DOX) supplemented food (Provimi Kliba) or control food was administered. Tumor dimensions were measured every one or two days in right angles using a digital caliper; total tumor volumes were determined by measuring two diameters (d1, d2) ($V = (4/3) + r^3$; $r = (d1 + d2)/4$). Mice were euthanized when reaching a tumor volume of 1'000 mm^3 and tumors extracted for further analysis. Autopsy was performed on each mouse to exclude the presence of major general toxicity events. Xenograft studies were approved by the Cantonal Veterinary Office of Zurich, and all animal care was in accordance with the existing Swiss legislation and guidelines.

Immunohistochemistry

Tumors obtained by dissection from sacrificed mice were fixed in PFA, and immunohistochemical analysis was done as described before (Amstutz, 2008). H&E, cleaved Caspase3 and Ki67 were stained. For quantitative evaluation, the number of positive cells was counted in five randomly selected visual fields in non-necrotic areas of the tumor using Image J software.

Mass spectrometry

After immunoprecipitation of Flag-EWS/FLI1, protein was concentrated to 15 µl using a precipitation concentrating kit (Uppa-Protein ConcentrateTM kit (GBiosciences). Digestion was performed with 60 ng of chymotrypsin (Promega) at 37° C overnight. Peptides were purified using ZipTip C18 tips (ThermoFisher Scientific AG) by resuspending in 3% ACN, 0.1% TFA and eluted with 60% ACN, 0.1% TFA twice. Peptides were dried in a speed vacuum centrifuge, resuspended in 3% ACN, 0.2% formic acid and 10 mM citric acid solution and injected into the mass spectrometer LTQ-OrbitrapVelos ETD (Thermo Fisher Scientific, Bremen, Germany) coupled to an EksigentNanoLC-Ultra 1D plus (Eksigent Technologies, Dublin (CA), USA). Solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid, 99.9% ACN for channel B. Peptides were loaded on a self-made frit column (75 µm × 150 mm) packed with reverse phase C18 material (ReproSil-Pur 120 C18-AQ, 1.9 μm, Dr. Maisch GmbH)) and eluted at a flow rate of 250 nL/min by a gradient from 3 to 30% of ACN in 56 minutes. The following LC-MS/MS acquisition methods were performed: (a) Orbitrap full MS scan followed by collision-induced dissociation (CID) fragmentation performed on the twenty most intense signals per cycle; (b) Orbitrap full MS scan followed by electron transfer dissociation (ETD) fragmentation on the

ten most intense signals per cycle. Full-scan MS spectra (300–1700 m/z) were acquired in profile mode at a resolution of 30000 at 400 m/z, an accumulation gain control (AGC) of $1x10^6$ and a maximum injection time of 200 ms. The AGC values for MS/MS analysis for both CID and ETD experiments were set to $1x10^4$ (ion trap detection, 100 ms injection time). The isolation width was set to 2 amu and 4 amu for CID and ETD experiments, respectively. All the experiments were recorded in data-dependent manner from signals above a threshold of 1000. The CID normalized collision energy was set to 35% and the maximum injection was 50 ms.For ETD experiments, the ETD anion target value was set to $1x10^5$ and the activation time to 50 ms. Charge-state dependent ETD reaction times were enabled, setting a reference value of 100 ms for doubly charged peptides. A supplemental activation with 25% normalized collision energy was always enabled. Precursor masses already selected for MS/MS were excluded for further selection for 45s. The exclusion window was set to 10 ppm and the exclusion size was limited to a maximum of 500 entries. Charge state screening was enabled and singly charge states were rejected. The samples were acquired using internal lock mass calibration on m/z 429.088735 and 445.120025.

Database search and protein identification

The raw files from the mass spectrometer were converted into Mascot generic files (mgf) with Mascot Distiller software 2.4.3.3 (Matrix Science Ltd.,London, UK) and peak lists searched using Mascot Server 2.3.02 against the forward UniProtKB/Swiss-Prot database for human, concatenated to a reversed decoyed FASTA database consisting of a total of 135,183 proteins and 260 common protein contaminants (NCBI taxonomy ID 9606, release date 2012-04-12). The protein sequence of EWS/FLI1 was included in the database. The parameters for precursor tolerance and fragment ion tolerance were set to ± 5 ppm and ± 0.8 Da, respectively. Phosphorylation (S, T, Y) and oxidation (M) were set as variable modifications. Peptides having an expectation value higher as 0.05 and/or a Mascot score lower as 20 were excluded. All the spectra of phosphorylated peptides were manually validated.

Results

Identification of post translational modifications in EWS-FLI1

Mass spectrometry (MS) is a powerful tool for identification of post translational modifications with high precision. To ensure identification of most modified peptides of

EWS-FLI1, we included different approaches such as various cellular expression systems, trypsin and chymotrypsin digestions and measurements on two different LC-MS/MS instruments (**Fig. 1A**). First, we transiently expressed the fusion protein in HEK293T (human embryonic kidney) and A673 ES cells, followed by purification via a 3X Flag-tag with anti-Flag antibody. Second, EWS-FLI1 was prepared from RD ES cells that stably express 3X Flag-EWS-FLI1 in which the fusion protein is expressed at levels more similar to the endogenous protein (**Suppl. Fig. 1**). The purified fusion protein was then digested with either trypsin (in gel digestion) or chymotrypsin (in solution digestion) and purified peptides were analysed by LC-MS/MS using LTQ-Orbitrap (data not shown) and LTQ-Orbitrap Velos. Coverage of the fusion protein by MS was between 75 - 85 % when chymotrypsin was used for digestion (**Fig. 1B**) and 30 % only with trypsin since there are no trypsin sites present in the EWS precluding analysis of peptides from this region. Independently of the cell line used for expression, we identified four peptides as being phosphorylated (**Fig. 1C**), including the phospho-T79 residue which was previously described [18]. In addition, we identified the novel site S287 as being phosphorylated in all experiments and two additional phosphorylated peptides containing S344-T347 and T470-S471-T473 which were not covered when RD ES cells were used. Except T79, all potential phosphorylation sites are present in the FLI1 part of the fusion protein (**Fig. 1D**). Whereas we could clearly identify S287 as being phosphorylated (**Fig. 1E**), for the other two peptides containing S344-T347 and T470-S471-T473 it was not possible to assign phosphorylation to a single unique residue (**Suppl.Fig. 1B-D**). Therefore, all of these were considered as candidates.

In addition to phosphorylation, we searched for other post translational modifications such as methylation and acetylation. The only residue found methylated was R484 (**Fig. 1C**). Peptides containing this site mono- and di-methylated were identified from all purification strategies. However, the function of R384 methylation was not further investigated. In contrast, no evidence for acetylation was found under the conditions used here. Nonetheless, it might be possible that acetylation of EWS-FLI1 requires stimulation of the cells [16].

To determine if sites phosphorylated in EWS-FLI1 were also modified in wild type FLI1, we expressed and purified 3X Flag-FLI1 in HEK293T cells. Protein was digested with chymotrypsin and the peptides analysed by MS as before. Indeed, the residue corresponding to S287 (**S241**) was found phosphorylated in the full length FLI1 but not the residues equivalent to T470-S471-T473 whereas the peptide containing S344-T347 was not covered. An additional phosphorylation site was found in wild type FLI1 (**S39**) that it is not present in the fusion protein (**Fig. 1F**).

To validate the presence of S287 phosphorylation in additional ES cells, we generated a phospho-specific S287 peptide antibody. After validating its specificity for the phosphorylated peptide (**Suppl. Fig. 2A**), endogenous EWS-FLI1 was immunoprecipitated using anti-FLI1 antibody from four different ES cell lines (A673, SKNMC, RD ES, TC71) and analysed by western blotting (**Fig. 2G**). In all cells, the presence of S287 phosphorylation under non stimulating conditions could be confirmed.

Hence, these experiments identified a novel site of phosphorylation in EWS-FLI1 under non stimulating conditions, therefore suggesting that the fusion protein is constitutively phosphorylated. Since the site is conserved in wild type FLI1, it might play a general role in regulating the activity of these proteins.

EWS-FLI1 target genes are modulated by phosphorylation

In a first approach to investigate the function of EWS-FLI1 phosphorylation we compared the transcriptional activity of wild type and phosphorylation-deficient mutants of the fusion protein by monitoring the mRNA expression levels of its target genes in a heterologous cell background. We mutated all phosphorylation sites identified in the FLI1 region (S287-S344-T347-T470-S471-T473) simultaneously to alanine to prevent phosphorylation (Pmut). Then, we retrovirally transduced RD (embryonal Rhabdomyosarcoma) cells with Pmut and wild type EWS-FLI1. After 3 days of transduction, upregulation of EWS-FLI1 target genes could clearly be measured when wild type fusion protein was expressed whereas Pmut expression stimulated target genes to only about 50% of the wild type levels (NKX2.2, NROB1, IGF1, SOX2 and NPY1R) (**Fig. 2A-B**), whereby expression levels of wild type and Pmut proteins were comparable. These data demonstrate that phosphorylation in general is important for full transcriptional activity of EWS-FLI1. Next, we used a similar approach to investigate single mutants. Interestingly, from all alanine mutants tested (data not shown), S287A was the only one showing decreased activity compared to wild type, despite similar protein expression levels (**Fig. 2A-B**). It should be noted that not all target genes tested were affected equally. For example, NGFR expression increased by 30% with both wild type and S287A mutant, and also for SOX2 no significant difference was observed, suggesting that expression of NGFR and SOX2 seem to be independent of this phosphorylation site. Thus, phosphorylation at S287 is required to regulate a subset of EWS/FLI1 target genes in heterologous rhabdomyosarcoma cells.

Cell growth of ES cells is affected by EWS/FLI1 phosphorylation

Next, we wanted to assess the importance of S287 phosphorylation for ES oncogenicity. Since the cell of origin of ES is still under debate and the cellular environment may affect the behaviour of the fusion protein, investigating the role of phosphorylation should ideally be performed in ES cells [21-27]. Thus, we developed a stable cell line expressing an shRNA to inducibly silence endogenous EWS/FLI1 in the presence of doxycyclin (A673-shEWS/FLI1). The shRNA construct targets the 3´UTR of FLI1 enabling simultaneous overexpression of wt or mutant Flag-EWS/FLI1 lacking the 3'UTR sequences from a second lentiviral construct as verified by western blotting (**Fig. 2D, E**). We generated A673-shEWS/FLI1 cells expressing GFP, Flag-EWS/FLI1, Flag-EWS/FLI1(S287A) or a previously described dominant negative mutant Flag-EWS/FLI1(R386N) [28, 29] to study their oncogenic properties. First, we assessed cell growth rates after daily addition of doxycyclin (100 ng/ml) to the medium and used uninduced A673-shEWS/FLI1 and A673-scramble cells as control. As expected, silencing of endogenous EWS/FLI1 and expression of GFP or inactive Flag-EWS/FLI1(R386N) led to a clear reduction of cell growth (**Fig. 3A**) which could be rescued by overexpression of Flag-EWS/FLI1. Interestingly, expression of the Flag-EWS/FLI1(S287A) mutant lacked the capability to rescue cell growth, indicating that S287 phosphorylation is indeed important for normal cell growth. Addition of doxycyclin did not affect cell growth of the A673-scramble cells. These results could also be confirmed by BrdU incorporation and crystal violet stainings of the same cell lines (**Fig. 3B**).

Cell cycle progression depends on S287 phosphorylation

After observing that lack of EWS/FLI1 phosphorylation impairs cell growth rate, we next investigated if the modification is important for cell cycle progression of A673 cells. To this end, we induced silencing of endogenous EWS/FLI1 for 4 days and collected the cells for flow cytometry analysis after staining with propidium iodine to compare the cell cycle pattern of wt and EWS/FLI1(S287A) cell lines. The results shown in Figure 3C indicate that depletion of endogenous EWS-FLI1 by doxycycline addition alters the cell cycle by increasing the sub-G1 population from 7% to 20% (**Fig. 3C and table**) which can be rescued by expression of wt EWS/FLI1 back to 8%. Rescue was not possible with expression of EWS-FLI1(R386N) and remained at 17%. Importantly however, expression of Flag-EWS-FLI1(S287A) increased the fraction of cells in G1 from 30% in controls to 54% without increasing the sub-G1 population.

Taking together, lack of phosphorylation at the S287 site impairs cell cycle progression, possibly since expression of a cell cycle regulator might depend on full EWS-FLI1 activity.

Clonogenic capacity of ES cells depends on EWS-FLI1 phosphorylation

The clonogenic capacity of ES cells is abolished when endogenous EWS-FLI1 is silenced and can be rescued when wild type Flag-EWS-FLI1 is introduced in the same cell line (**Fig. 3D**). Therefore, we next investigated whether expression of the EWS-FLI1(S287A) mutant would be able to generate the same number of colonies as the wt fusion protein. Indeed, expression of the S287A mutant shows a decrease in the number of colonies compared to wt EWS/FLI1 (**Fig. 3D**).

Hence, also the clonogenic capacity of ES cells was reduced by lack of phosphorylation.

Phosphorylation of S287 is critical for efficient DNA binding

Phosphorylation of transcription factors is a well-known mechanism to control nuclear transport. Hence, we wondered whether phosphorylation would be important for nuclear localization of EWS-FLI1. We transciently expressed wt and Flag-EWS-FLI1(S287A) in A673 cells and monitored the cellular localization of the fusion protein by immunofluorescence using anti-Flag antibody. Both proteins were similarly localized in the nucleus indicating that S287 phosphorylation does not regulate nuclear entry (**Suppl. Fig.2C**).

Next, we investigated whether lack of S287 phosphorylation might affect protein stability. For this, we use a Global Protein Stability approach ([30] and Gierisch et al. under revision). HEK293T cells were transduced with a reporter construct DsRed-IRES-EGFP in which EGFP is fused to EWS-FLI1 or EWS-FLI1 (S287A). The ratio EGFP/DsRed represents a measure for protein stability and was determined by FACS. The estimated half-life of both proteins was between 1h and 4h, and no differences were observed indicating that also stability is not affected by phosphorylation (**Suppl.Fig.2D**).

Finally, we investigated the DNA binding capacity of EWS-FLI1(S287A) compared to wt using an electrophoretic mobility shift assay (EMSA). In order to establish the assay, we first transiently expressed Flag-EWS-FLI1, Flag-EWS/FLI1(Pmut), Flag-EWS-FLI1(R386N), and Flag-empty in HEK293T cells, lysed the cells 48 hrs post-transfection and incubated crude extract with a biotinylated oligonucleotide containing the characteristic DNA binding motive GGAA [31] present in the promoter of EWS-FLI1 target genes [32] (**Suppl. Fig. 3A**). The results indicate that DNA binding capacity of EWS-FLI1(Pmut) was reduced compared to wt

EWS-FLI1 (**Suppl. Fig. 3A, lines 2 and 5**). Therefore, we carried out the same assay with the single mutant Flag-EWS-FLI1(S287A) (**Fig. 4A**).

The complex generated in the presence of wt EWS-FLI1 and biotinylated oligonucleotide could be displaced by the presence of excess non-biotinylated oligonucleotide (**Fig. 4A, lines 2, 3**) as well as by pre-incubation with an anti-FLI1 but not an unrelated antibody (**Fig. 4A, lines 4, 5**). Importantly, crude extract from cell transfected with Flag-EWS-FLI1(S287A) generated a significantly reduced band compared to wt EWS-FLI1(**Fig. 4A line 6**), whereas EWS-FLI1 proteins were expressed at equal levels (**Fig. 4B**). These results indicate that phosphorylation of S287 might affect DNA binding of EWS/FLI1 in heterologous cells.

We next used crude extracts from ES cells (A673-shEWS-FLI1 and Flag-EWS-FLI1(S287A) in presence or absence of doxycycline to determine the DNA binding capacity by EMSA directly from ES cells. Without doxycycline induced depletion of endogeneous EWS-FLI1, a shift was observed when no specific competitor was added (**Fig. 4C, lines 2, 4, 9, and 11**). In the presence of doxycycline, all bands showed reduced intensity (lines 5-8). Importantly, when extract was generated from EWS-FLI1(S287A) expressing cells, no shift was detected at all under treatment with doxycycline depleting the wild type fusion protein (**Fig. 4C, lines 12, 13, and Fig. 4D**). Therefore, EWS-FLI1(S287A) showed reduced DNA binding compared to wt protein also directly in ES cells.

We expected that a decrease in DNA binding seen with EWS-FLI1(S287A) would also lead to reduced levels of target genes. Therefore, we measured the expression of target genes in A673-shEWS-FLI1 cells by qRT-PCR. Silencing of endogenous EWS-FLI1 leads to reduced mRNA levels of activated target genes (NKX2.2, NROB1) and an upregulation of the repressed targed gene PHLDA1 (**Fig. 4E**) which could be rescued by expression of wt Flag-EWS-FLI1. Importantly, expression of EWS-FLI1(S287A) was not able to rescue target gene expression. These results were confirmed also in a second cell line, SKNMC, using the same approach (**Suppl. Fig. 3B**). Taken together, EWS-FLI1(S287A) showed reduced transcriptional activity compared to wt EWS-FLI1 likely due to reduced DNA binding affinity.

EWS-FLI1(S287A) cells show reduced growth in xenografts

Finally, we assessed growth of mutant EWS-FLI1(S287A) ES cells directly *in vivo*, since we observed a reduced growth rate compared to the wt already *in vitro*. Therefore we engrafted $5x10^6$ A673-shEWS-FLI1 cells into 8-12 weeks-old NOD/Scid il2rg-/- mice. For each condition, namely EWS-FLI1(S287A), EWS/FLI1(wt), and GFP expressing cells, 10 mice were engrafted (9 for GFP control), and mice were fed either with DOX supplemented or

control food once tumors were palpable. Out of total 29 mice only 5 did not develop tumors and therefore 3 or more mice per condition could be assessed. We observed rapid tumor growth in untreated control mice similar for all three constructs that reached the maximum volume within 14 days (**Fig. 5A**). Tumors of mice engrafted with GFP cells that expressed only the endogenous EWS-FLI1, took 10 days more to reach comparable volumes to cells expressing wt. However, when doxycycline supplemented food was provided and endogenous EWS-FLI1 was depleted, similar differences in tumor growth were observed as *in vitro*. Silencing of the fusion protein in the mice engrafted with GFP control cells led to tumor regression within 10 days of treatment. While mice engrafted with EWS-FLI1(wt) cells showed even faster tumor growth compared to control, tumors expressing the S287A mutant grew significantly slower and reached maximal tumor size only after 60 days.

To investigate the possible cause of tumor growth retardation in EWS-FLI1(S287A) cells, we performed immunohistochemical analysis for the proliferative marker Ki67 and for the apoptosis effector cleaved caspase 3 (**Fig. 5B**). Indeed, we observed a 50% reduction in Ki67 positive cells upon silencing of endogenous EWS-FLI1 (**Fig. 5C**), and no significant increase in cleaved caspase 3 positive cells. These results therefore suggest that cells overexpressing EWS-FLI1(S287A) are proliferating with a lower rate, as previously observed *in vitro*.

After 30 days, tumors recurred even under treatment and mice had to be sacrificed when reaching 1000 mm^3 tumor volume. To explain the relapsed tumors observed in GFP DOX treated mice, we measured endogenous EWS-FLI1 protein levels (**Fig. 5D**). In relapse tumors of mouse #4 and #5, we observed an increase in EWS-FLI1 expression up to three fold compared to non-treated mice (#1,#2,#3), thus indicating that relapse tumors likely are due to re-expression of endogenous EWS/FLI1 while for mouse #6 which was treated only 4 days with DOX supplemented food, EWS-FLI1 levels were lower by 40%, indicating that silencing of endogenous EWS-FLI1 expression was achieved during the initial phase of the experiment.

Taken together, we conclude that replacement of the wild type fusion protein by the single mutant EWS-FLI1(S287A) in ES cells triggers slower tumor growth indicating that phosphorylation at this site is important for full activity and tumorigenesis of the oncogenic EWS-FLI1 protein.

Discussion

Pediatric sarcomas represent rare tumor types with still unacceptable low cure rates. ES is a malignancy driven by an oncogenic fusion protein, EWS-FLI1, which is the unique genetic abnormality identified in the majority of these tumors. Studies to date reveal that EWS-FLI1

is both an activator and repressor of a large set of genes, contributing to oncogenesis in ES. EWS-FLI1 fusion protein represents a unique molecular target for tumor cell specific therapy. However, the molecular mechanism regulating EWS-FLI1 activity remains unclear. Identification of PTMs of EWS-FLI1 that influence its oncogenicity will likely facilitate the development of novel targeted therapies.

In this study, through a combination of MS and cellular assays, we identified novel phosphorylation sites EWS-FLI1 and determined the function of phosphorylation in terms of oncogenicity of EWS-FLI1. We probed by MS the phosphorylation sites present on EWS-FLI1 under non stimulating conditions using three different ES cell lines. A previous study, using a phospho-specific antibody, demonstrated that T79 (in the EWS domain of the fusion protein) is phosphorylated upon DNA damage [18]. We have also clearly identified phosphorylation at T79, even in unstimulated cells. Furthermore, we identified a novel phosphorylation site present in the FLI1 region of the fusion protein, S287, that influenced the oncogenicity of EWS-FLI1. Interestingly, even though the residue S287 does not directly lie in the DNA binding domain of FLI1, its mutation to alanine decreases DNA binding as demonstrated by EMSA in both HEK293T and A673 ES cells. The immediate consequence of decreasing DNA binding is a decreased expression or repression of EWS-FLI1 target genes. In our cellular model, where endogenous EWS-FLI1 was knocked down and S287A was ectopically expressed in A673 cells, we detected a decrease of NKX2.2, NROB1 and an increase in PHLDA1 mRNA levels. This result was confirmed in a second ES cell line (SKNMC cells). We investigated next whether lack of phosphorylation at S287 had additional consequences on the biology of EWS-FLI1. While neither the cellular localization nor the protein stability was affected, cell growth of A673 expressing EWS/FLI1(S287A) was clearly decreased compared to wt EWS-FLI1. This finding is supported by an increase in the fraction of cells in the G1 phase of the cell cycle as identified by flow cytometry. In a recent publication [33], incubation of A673 cells with the compound Englerin A inhibits colony formation and decreases DNA binding. This effect was explained by a decrease in phosphorylation of Ser residue/s in EWS-FLI1, but the S266 site of phosphorylation identified in this study is not present in type 1 fusion protein used here. Nevertheless, we observed diminished colony formation and decreased DNA binding in this work for EWS-FLI1(S287A), which is similar to what has been described by Caropreso et al. Using our phospho-specific S287 antibody, we confirmed the presence of phosphorylation in four ES cells A673, SKNMC, TC71 and RD ES Ewing cells, however we were unable to detect any

significant decrease of phosphorylation at this site when treating cells with either Englerin A or depleting PKCβI (data not shown).

Together however, these findings underscore the importance of phosphorylation for DNA binding and subsequent oncogenic activity of EWS-FLI1. Further investigations are needed to elucidate the upstream protein kinase(s) responsible for phosphorylating the different residues in EWS-FLI1.

Figure legends

Figure 1: EWS-FLI1 post translational modifications identified by mass spectrometry. (A) Schematic representation of the approach to identify PTMs in EWS-FLI1. (B) Sequence of EWS-FLI1 showing the peptides covered by MS after chymotrypsin digestion. (C) Chymotryptic peptides modified in EWS-FLI1 from different cell lines indicated are listed. S/T phosphorylation and R methylation are underlined. (D) Schematic representation of PTMs in EWS-FLI1 identified in this study. (E) Spectra of the modified S287 residue assigning the phosphorylation site. (F) Phosphorylation sites (underlined) identified in full length FLI1 in HEK293T cells. (G) Western blot of EWS/FLI1 showing phosphorylation of S287 in different Ewing cell lines. After immunoprecipitation of endogenous EWS/FLI1 with anti-FLI1 antibody, phosphorylation of S287 was visualized using the specific anti phospho S287 antibody.

Figure 2: EWS-FLI1 target genes are modulated by phosphorylation. (A) Equal protein expression level of wt and mutants EWS-FLI1 in RD cells corresponding to the samples used for the qRT-PCR assay shown in B. (B) mRNA level of EWS-FLI1 target genes was measured by qRT-PCR in RD cells overexpressing wt and mutant EWS-FLI1. Pmut refers to simultaneous mutation of S287-S344-T347-T470-S471-T473 to alanine. Data are shown as geometric mean of 3 independent experiments performed in triplicates; 95% confidence interval. All values were normalized to GAPDH and shown in relation to wt EWS-FLI1 which was set to 100% (C) Schematic representation of the cellular system used in this study to test the biological effect of EWS/FLI1 mutants. Red constructs indicate ectopic expression. (E) Western blot of lysates from A673 cells with knock down of endogenous EWS-FLI1 after doxycycline addition for 48hrs in the medium and simultaneous ectopic expression of either GFP, Flag-EWS-FLI1 (EF) or mutated Flag-EWS-FLI1(S287A). Blot was probed with an anti-FLI1 or anti-Flag antibody. Tubulin was monitored as loading control.

Figure 3: Biological effects of EWS-FLI1(S287A) *in vitro*. (A) Cell growth of indicated A673-shRNA cell lines. Cells from 2 independent wells were trypsinized, collected and counted for each time point. Average of three independent experiments performed in duplicates is shown. (B) BrdU incorporation and crystal violet staining of A673-shRNA cell lines after 3 days of doxycycline incubation. Each untreated cell line was considered as 100 %. The average of a triplicate is shown. (*C*) Cell cycle progression of A673-shRNA cell lines measured by FACS after propidium iodine incubation and 4 days of treatment of cells with 0.1 µg/ml doxycycline. Lower panel shows exact quantification. (D) 500 cells/well A673-shRNA cells were seeded in 6 well plates, in triplicates. Doxycycline was added daily for 21 days. Colonies were stained with crystal violet and number of colonies formed quantified. * indicates significance $p<0.05$.

Figure 4: DNA binding affinity depends on S287 phosphorylation. (A) EMSA assay in HEK293T cells overexpressing wt and mutants EWS-FLI1 as indicated. (B) Western blot of extracts isolated from HEK293T cells used for EMSA and probed for EWS-FLI1 and mutated EWS-FLI1(S287A) using both anti-Flag and anti-FLI1 antibodies. (C) EMSA assay with extracts isolated from A673-shRNA cells in the absence or presence of doxycycline. (D) Western blot of extracts isolated from A673-shRNA cell lines used for EMSA and treated with doxycycline for 48hrs. (E) qRT-PCR with primers detecting target genes NKX2.2, NROB1 (left panel), and PHLDA1 (right panel) after 2 days of doxycycline treatment of A673-shRNA cell lines. Values of untreated cells were set at 100%. A representative experiment is shown.

Figure 5: Tumor growth of EWS-FLI1(S287A) *in vivo*.

(A) Growth rate of A673 shEWS-FLI1 xenograft tumors in NOD/Scid il2rg-/- mice ($n\geq3$). Measurements were normalized to the first value set to 100 mm^3. (*B*) H&E, cleaved caspase 3 and Ki67 staining of xenograft tumor sections, isolated from mice once tumour reached the maximal tumour volume allowed. (*C*) Quantification of Ki67 positive cells counted using Image J software. More than 200 cells were counted in each section and values represent the average of 5 different sections. (D) Western blot of EWS-FLI1 protein from extracts of tumors isolated from control GFP mice and relapse tumors. Mice #4 and #5 presented relapse tumours whereas mouse #6 was treated only four days with Doxycliclin supplemented food.

Suppl. Figure 1: Phosphorylation sites of EWS-FLI1 identified by MS. (A) Western blot showing the EWS-FLI1 protein expression level in wt RD ES cells and stably expressing Flag-EWS-FLI1 RD ES cells used for MS. (B-D) Spectra of the modified residues assigning the phosphorylation site.

Suppl. Figure 2: Characterization of the phospho-specific S287 antibody and determination of cellular localization and protein stability of EWS-FLI1(S287A). (A) Phospho S287 antibody recognizes wt Flag-EWS-FLI1 immunoprecipitated from HEK293T cells but not the alanine mutant S287A (left panel). Binding of the phospho-specific antibody is displaced in the presence of excess of the phospho-peptide P-S287 used as immunogene (middle panel). Binding of the phospho-specific antibody to wt EWS-FLI1 cannot be displaced in the presence of excess of the nonphospho-peptide containing the original S287 residue (right panel). (B) Western blot of A673-shRNA cell lines after depletion of endogenous EWS-FLI1 by treatment with doxycycline. (C) Immunofluorescence of A673 cells overexpressing either Flag-EWS-FLI1 or Flag-EWS-FLI1(S287A) with anti-Flag antibody, costained with Dapi. (D) Stability of proteins was determined by Global Protein Stability. HEK293T were transduced for 72hrs with a reporter construct fused to EWS-FLI1 or degron motifs with half lives of 1h (d1), 4h (d4) and 24h (d24) and analyzed by FACS.

Suppl. Figure 3: Reduced transcriptional activity of EWS-FLI1(S287A) in SKNMC-shRNA cell lines. (A) EMSA assay from HEK293T cells overexpressing wt and mutants EWS-FLI1. (B) qRT-PCR with RNA isolated from SKNM-shEWS-FLI1 cells after 2 days of treatment with doxycycline. A representative experiment is shown. Right panels shows a western blot of SKNMC-shEWS-FLI1 cells showing after doxycycline treatment for 48hrs.

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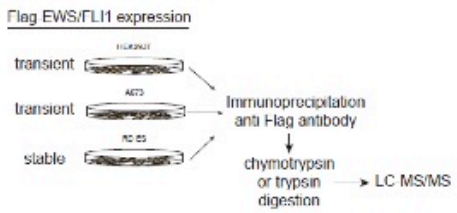
Figure 1

A



B

Matched peptides shown in **Bold Red**

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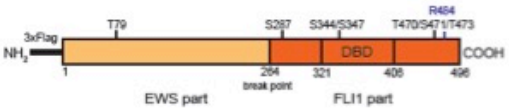
 51 PTQGYAQTTQ AYGQQSYGTY GQPTDVSYTQ AQTTATYGQT AYATSYGQPP
101 TGYTTPTAPQ AYSQPVQGYG TGAYDTTTAT VTTTQASYAA QSAYGTQPAY
151 PAYGQQPAAT APTRPQDGNK PTETSQPQSS TGGYNQPSLG YGQSNYSYPQ
201 VPGSYPMQPV TAPPSYPPTS YSSTQPTSYD QSSYSQQNTY GQPSSYGQQS
251 SYGQQSSYGQ QPPTSYPPQT GSYSQAPSQY SQQSSSYGQQ NPSYDSVRRG
301 AWGNNMNSGL NKSPPLGGAQ TISKNTEQRP QPDPYQILGP TSSRLANPGS
351 GQIQLWQFLL ELLSDSANAS CITWEGTNGE FKMTDPDEVA RRWGERKSKP
401 NMNYDKLSRA LRYYYDKNIM TKVHGKRYAY KFDFHGIAQA LQPHPTESSM
451 YKYPSDISYM PSYHAHQQKV NFVPPHPSSM PVTSSSFFGA ASQYWTSPTG
501 GIYPNPNVPR HPNTHVPSHL GSYY
```

C

Predicted mass	Observed mass	Residue number	Peptide sequence	Modification	HEK293T	A673	RD ES
1728.74	1728.74	71-86	GQPPTGYTTPTAPQAY	phosphorylation	identified	identified	identified
2802.33	2802.34	285-309	NKSPPLGGAQTISKNTEQRPQPDPY	phosphorylation	identified	identified	identified
1588.65	1588.64	335-348	ELLSDSANASCITW	phosphorylation	identified	identified	not covered
2954.39	2954.40	469-494	WTSPTGGIYPNPNVPRHPNTHVPSHL	phosphorylation	identified	identified	not covered
2702.37	2702.38	470-494	TSPTGGIYPNPNVPRHPNTHVPSHL	methylation	identified	identified	not covered
2716.38	2716.39	470-494	TSPTGGIYPNPNVPRHPNTHVPSHL	di-methylation	identified	identified	not covered

D



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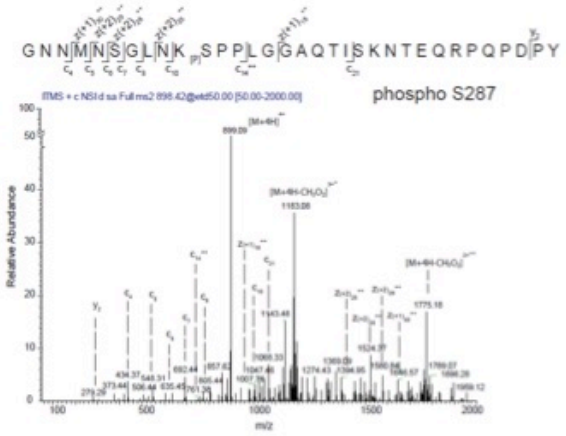


F

Predicted mass	Observed mass	Residue number	Peptide sequence	Modification
3589.66	3589.66	231-263	GAAAHLPKADMTASGSPDYGQPHKINPLPPQQEW	phosphorylation
3688.71	3688.71	24-57	GNNMNSGLNKSPPLGGAQTISKNTEQRPQPDPY	phosphorylation

G

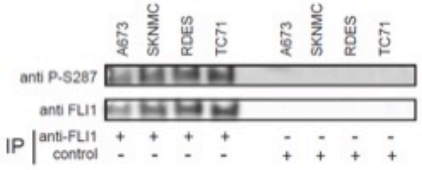
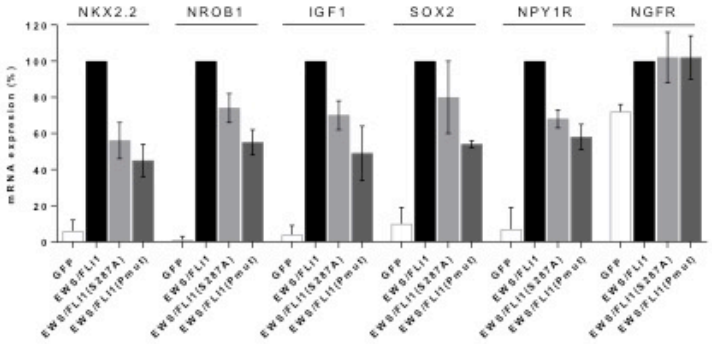


Figure 2

A

B



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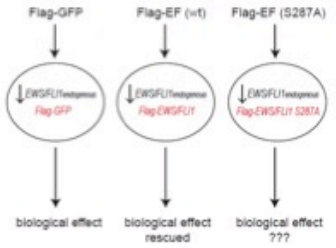

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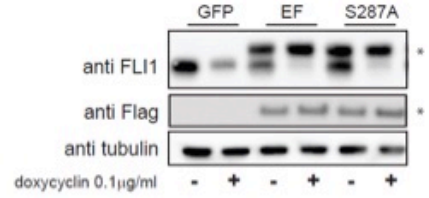

Figure 3

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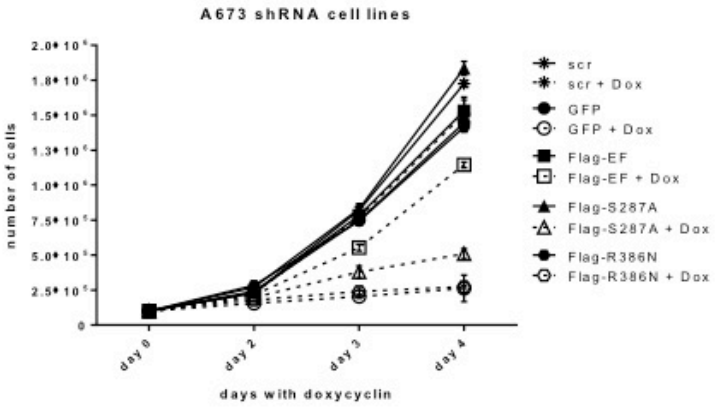

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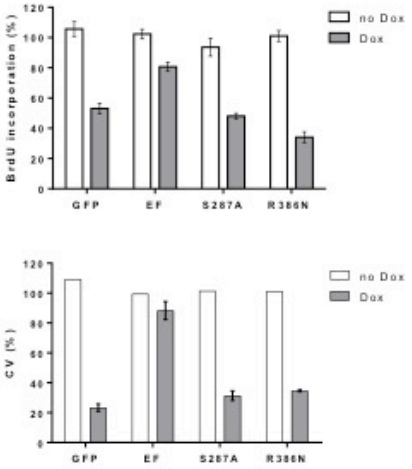

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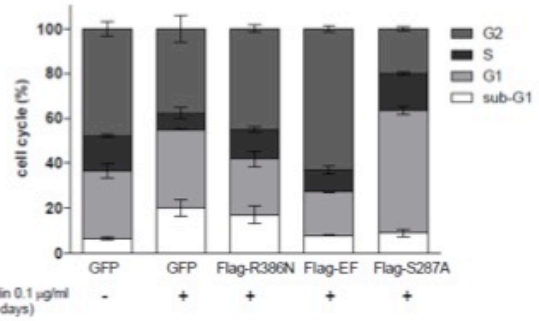


cell line	doxycyclin	sub-G1(%)	G1(%)	S(%)	G2(%)
GFP	-	6.6 ±0.7	29.9 ±3.2	15.7 ±0.6	47.7 ±3.1
GFP	+	20.1 ±3.7	34.8 ±0.3	7.3 ±2.5	37.7 ±5.9
Flag-EF	+	8.0 ±0.1	19.2 ±0.3	9.8 ±1.7	63.0 ±1.3
Flag-S287A	+	9.0 ±1.6	54.4 ±1.8	16.6 ±0.6	19.9 ±0.8
Flag-R386N	+	17.0 ±3.8	24.9 ±3.3	12.9 ±1.1	45.3 ±1.6

D

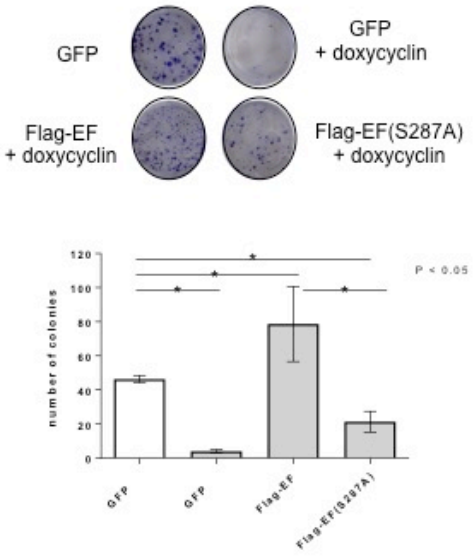
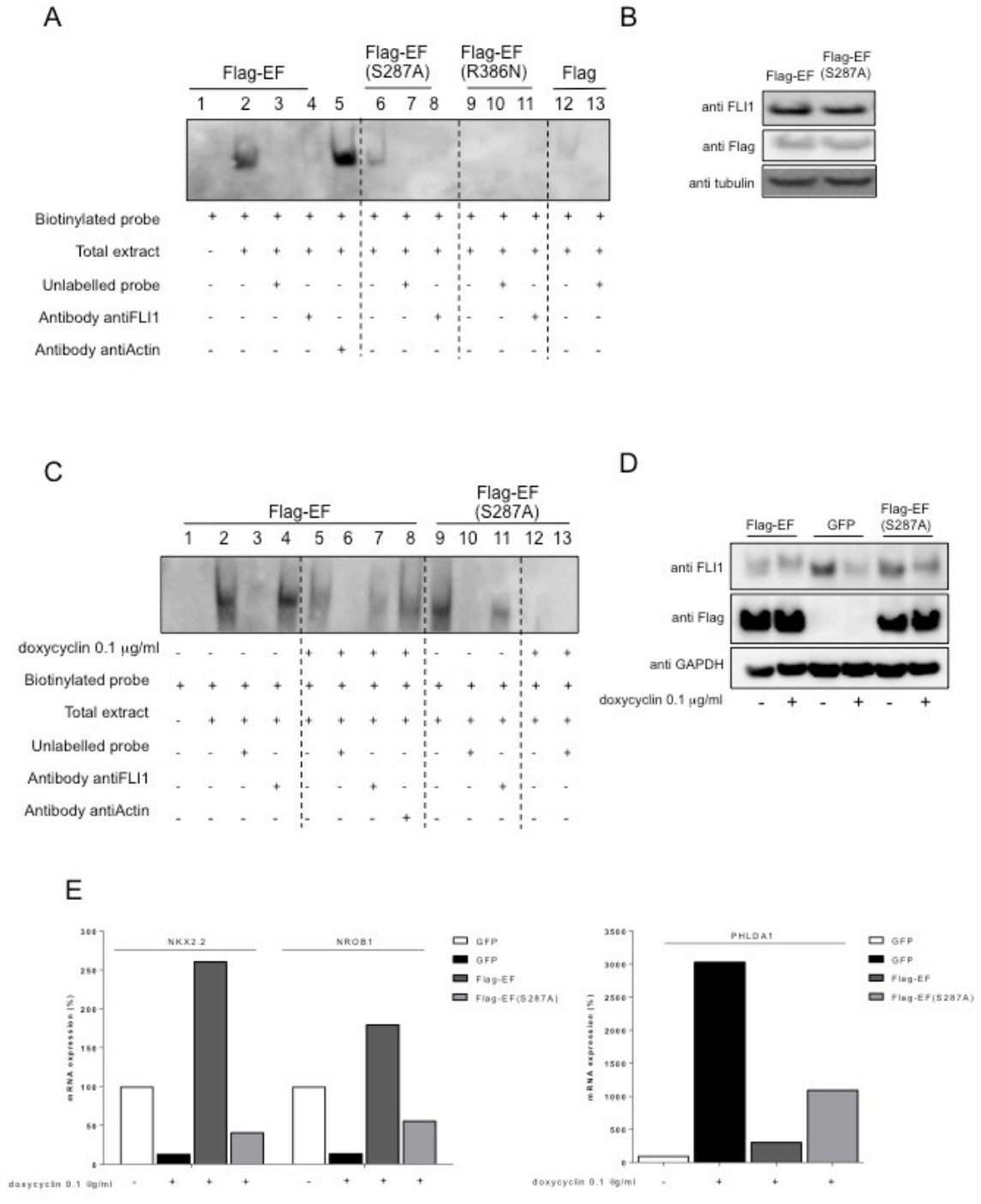


Figure 4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Flag-EF
Flag-EF (S287A)
Flag-EF (R386N)
Flag
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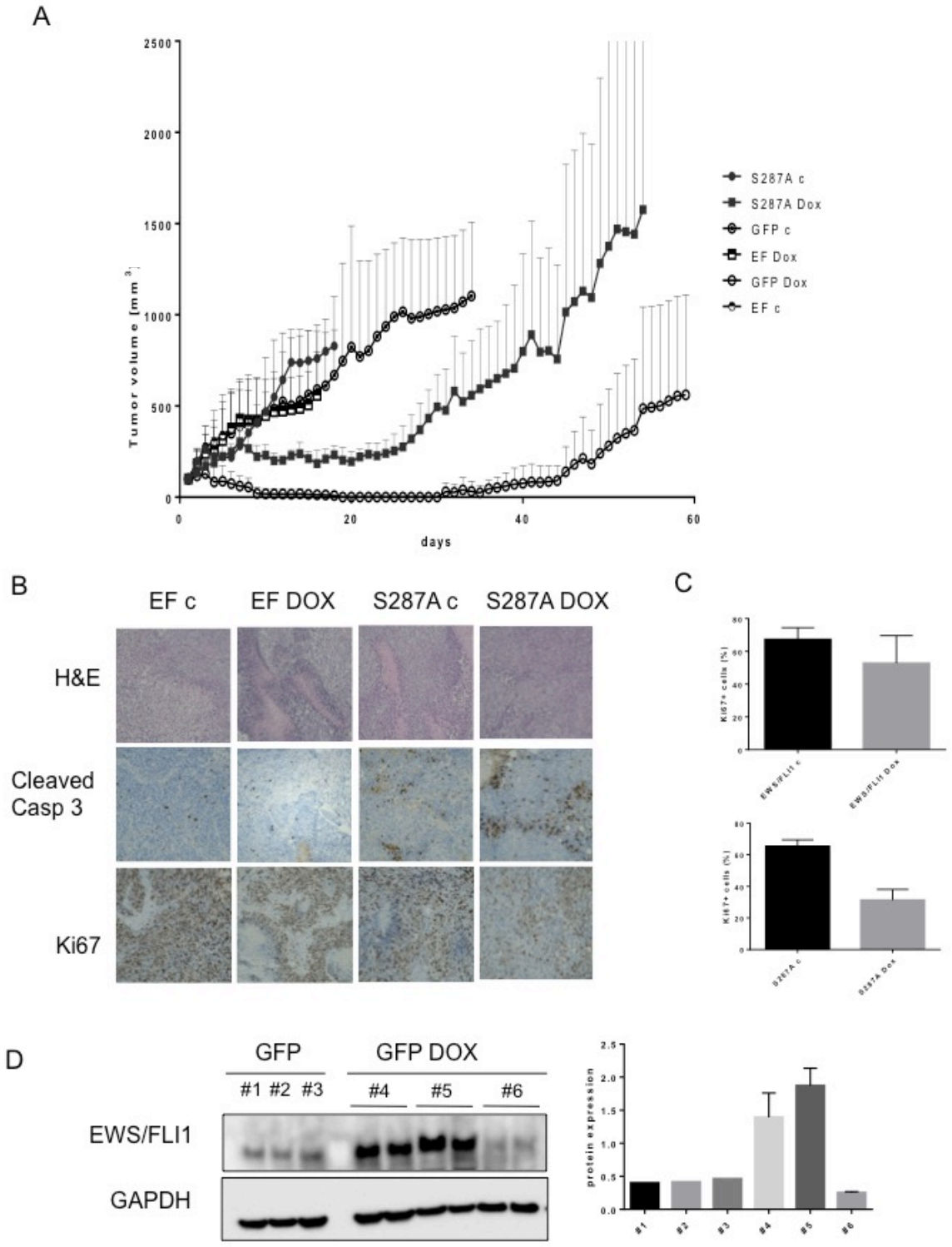
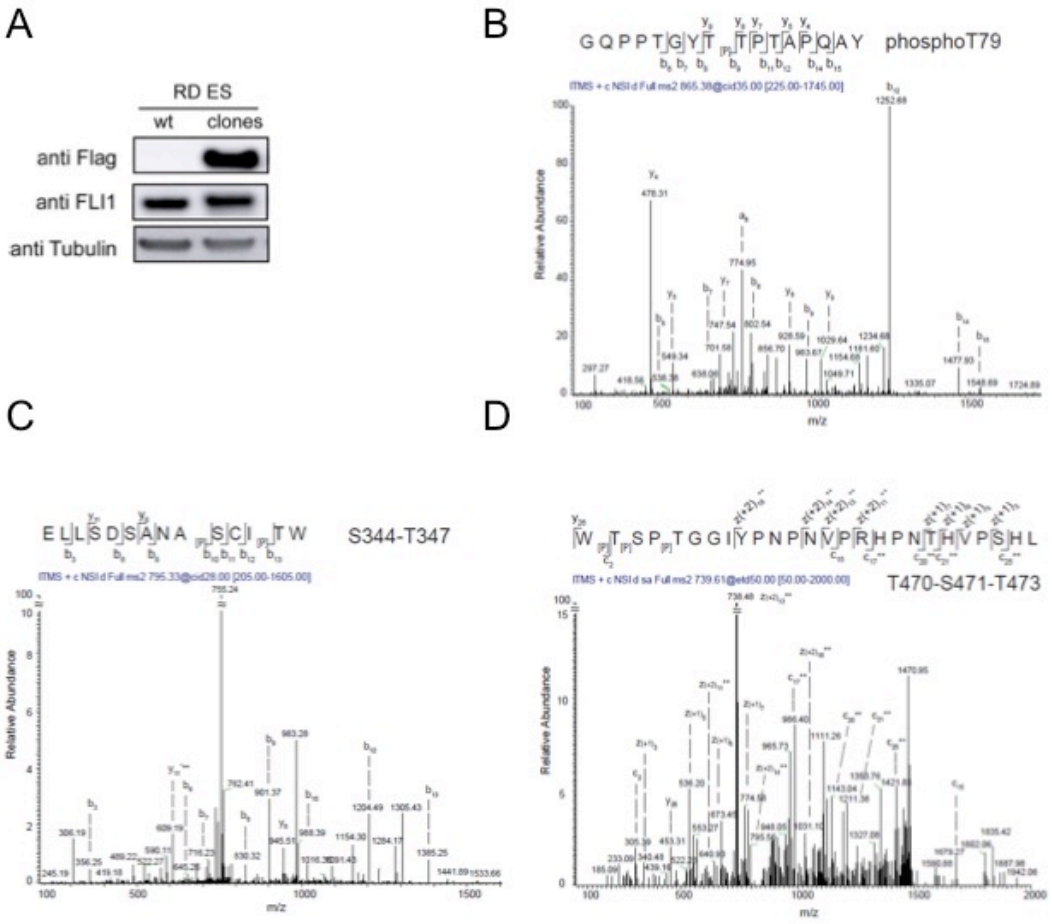
Biotinylated probe
Total extract
Unlabelled probe
Antibody antiFLI1
Antibody antiActin
B
Flag-EF
Flag-EF (S287A)
anti FLI1
anti Flag
anti tubulin
C
Flag-EF
Flag-EF (S287A)
1 2 3 4 5 6 7 8 9 10 11 12 13
doxycyclin 0.1 µg/ml
Biotinylated probe
Total extract
Unlabelled probe
Antibody antiFLI1
Antibody antiActin
D
Flag-EF
GFP
Flag-EF (S287A)
anti FLI1
anti Flag
anti GAPDH
doxycyclin 0.1 µg/ml
E
NKX2.2
NROB1
PHLDA1
GFP
GFP
Flag-EF
Flag-EF(S287A)
mRNA expression (%)
doxycyclin 0.1 µg/ml

Figure 5


A
Tumor volume [mm3]
days
S287A c
S287A Dox
GFP c
EF Dox
GFP Dox
EF c
B
EF c
EF DOX
S287A c
S287A DOX
H&E
Cleaved Casp 3
Ki67
C
D
GFP
GFP DOX
#1 #2 #3
#4
#5
#6
EWS/FLI1
GAPDH
protein expression

Suppl Fig.1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wt
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anti Flag
anti FLI1
anti Tubulin
B
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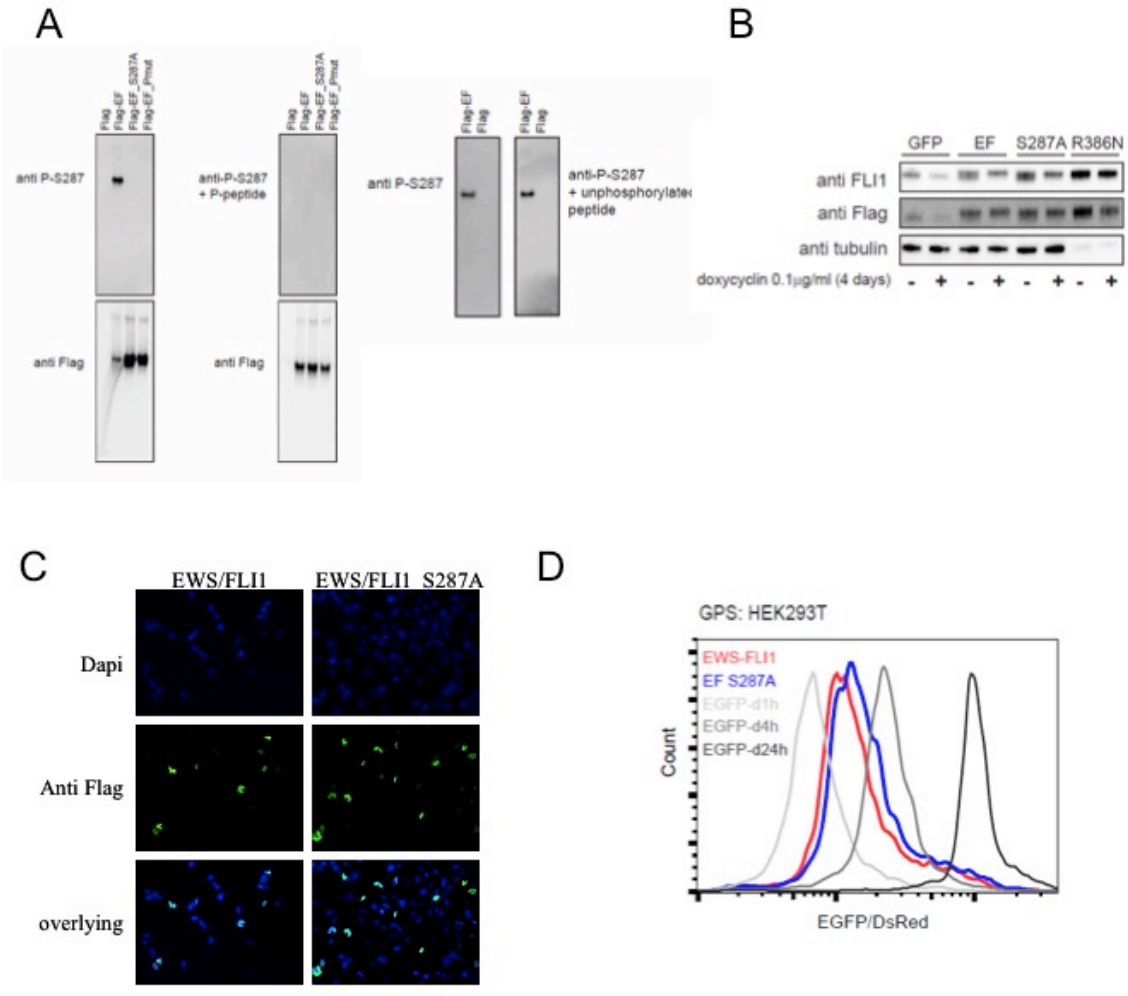
Relative Abundance
m/z
C
E L L S D S A N A [P] S C I [P] T W
S344-T347
Relative Abundance
m/z
D
W [P] T [P] S P [P] T G G I Y P N P N V P R H P N T H V P S H L
T470-S471-T473
Relative Abundance
m/z

Suppl Fig.2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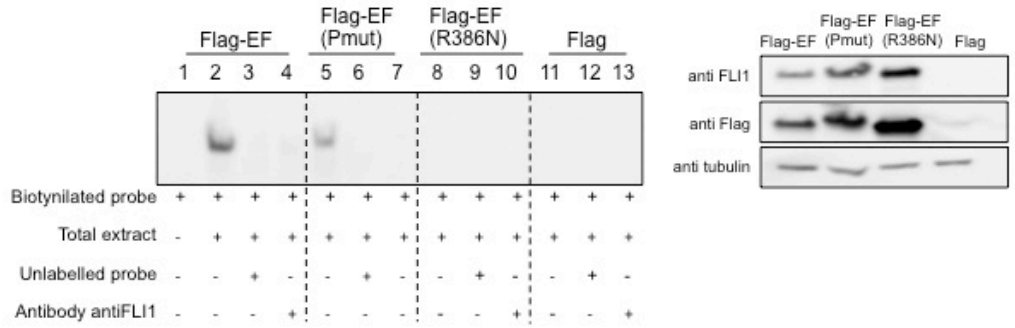
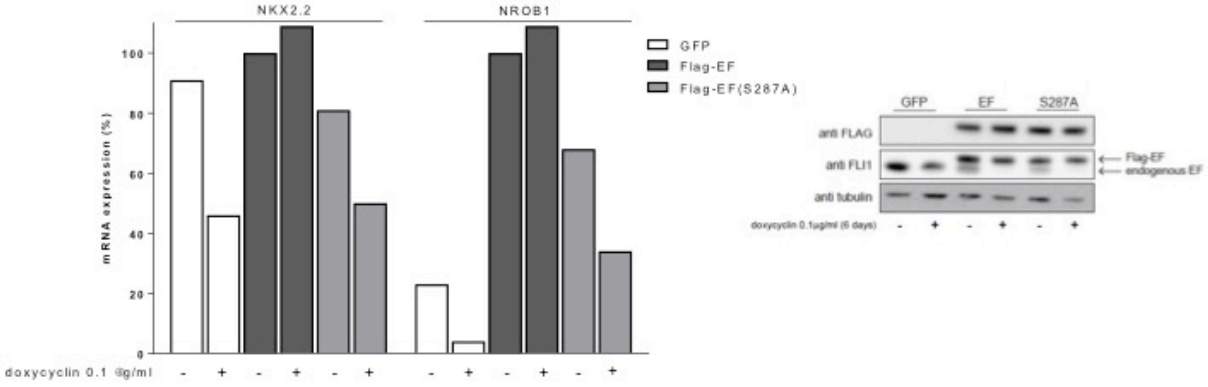
anti-P-S287
+ unphosphorylated
peptide
B
GFP
EF
S287A
R386N
anti FLI1
anti Flag
anti tubulin
doxycyclin 0.1μg/ml (4 days)
- + - + - + - +
C
EWS/FLI1
EWS/FLI1 S287A
Dapi
Anti Flag
overlying
D
GPS: HEK293T
EWS-FLI1
EF S287A
EGFP-d1h
EGFP-d4h
EGFP-d24h
Count
EGFP/DsRed

Suppl Fig.3

A



B



6. Discussion

Ewing sarcoma is a bone and soft tissue paediatric tumour that affects kids and young adults. Histologically it belongs to the small round blue cells tumours and is characterized by a large number of undifferentiated cells. Clinically, it is a very aggressive osteolytic tumour with an early tendency to metastasize. The main feature of Ewing sarcoma is the presence of a balanced translocation between chromosome 11 and 22 leading to the generation of a dysregulated transcription factor EWS/FLI1 in 85% of cases [75, 128, 436]. Indeed, the strong transactivation domain encoded by the EWSR1 gene is fused to the DNA binding domain of the ETS family member transcription factor FLI1. This fusion protein has been described as the main recurrent mutation in patient's samples and is therefore considered the driver of tumorigenesis [3, 66, 121-127]. It is generally thought that it drives the malignant phenotype through regulation of its target genes [115, 128, 147, 148, 172], nevertheless among them a specific driver has not been identified so far, suggesting that the fusion protein itself represents a very attractive target for therapy [137]. Being a transcription factor, EWS/FLI1 is generally considered undruggable [138]; indeed it lacks enzymatic activity and therefore alternative approaches are needed.

Current standard therapy is a combination of chemotherapy, radiotherapy and surgery and results in a survival rate of 70%. Unfortunately, patients with recurrent tumours or metastasis at diagnosis have a 5 year survival rate of 20% only. In addition, the current combination treatment does not include targeted therapies and recurrence or secondary malignancies often occur. Furthermore, the most commonly used drugs have been introduced 40 years ago and there is need to reduce mortality and morbidity rates. For these reasons, we focused here on targeting EWS/FLI1 fusion protein, expression or activity in Ewing sarcoma.

Therefore, the aim of my work was to identify a new targeted approach, extremely needed for therapy, capable of blocking the fusion protein activity or its expression thus we

decided to perform a drug screening with targeted compounds and then verify EWS/FLI1 gene expression and as well of its target genes.

Other group of researchers already tried and are trying to do the same through drug screening; indeed different compounds have been tested to identify one that is capable of reducing EWS/FLI1 activity [170, 293, 294]: AraC treatment for example attenuated an EWS/FLI1 signature by reducing fusion protein levels, thus resulting in a decreased cell viability, induction of apoptosis and abrogation of cell growth *in vivo* [241]. Also treatment with the antibiotic Mithramycin resulted in reduction of EWS/FLI1 activity with a consequent effect on target gene expression, and in suppression of tumour growth *in vivo* [294]. Recently new Mithramycin analogues are investigated and described to further decrease EWS/FLI1 activity with less toxicity for the cells [437].

From a previous drug screening performed in our laboratory, the kinase inhibitor Midostaurin (or PKC412) was identified to decrease Ewing cell viability. Indeed, the drug was also able to directly reduce EWS/FLI1 activity and subsequently induced apoptosis and inhibited tumour growth *in vivo* [170]. Unfortunately all these drugs alone are not sufficient for an efficient treatment and more combination studies with standard chemotherapeutics have to be tested.

From our drug screening of 153 targeted compounds, we identified PI3K inhibitors as modulators of EWS/FLI1 activity and surprisingly also of its gene expression. Among these, BEZ235, a dual mTOR-PI3K inhibitor, was selected for further studies. BEZ235 showed the strongest effects; it is a small molecule that competes with ATP for the binding to both mTOR's and PI3K's binding pockets therefore inhibiting their enzymatic activity.

The IGF1/PI3K/mTOR pathway is a major player in ES, since it is involved in cell proliferation and survival and it has been studied and described in detail. Most relevant aspect, its inhibition at different levels affects Ewing sarcoma cells growth [253, 258, 438].

As described in the introduction, treatment with Rapamycin, an inhibitor of mTORC1, resulted in EWS/FLI1 protein level reduction and therefore also its activity on target genes [276]; targeting directly IGF1 ligand or its receptor with compounds or specific antibody induced apoptosis in Ewing cells [230, 258, 259, 261]. Combination of IGF1 inhibitors with

Rapamycin [264, 274, 277-280] or with normal chemotherapeutics [262-264] resulted in a synergistic effect. Nevertheless rapid resistance occurs, suggesting the urgent need for a better understanding of the mode of action of the pathway and identification of possible crosstalks with other signalling.

Given the role of IGF1/PI3K/mTOR pathway in this pathology, we decided to further investigate the link between PI3K pathway and EWS/FLI1 regulation.

Treatment with BEZ235 was already known to reduce cell growth by blocking the cell cycle in G1 and to induce partial apoptosis *in vitro* and *in vivo* [292].

Since BEZ235 treatment was affecting EWS/FLI1 gene expression, we decided to focus on studying the promoter of EWSR1 gene, that is conserved in the fusion protein, in order to identify a possible transcription factor involved in its gene expression [429]. Through several deletion constructs using luciferase gene as reporter, we were able to identify a region of 20 bp, called Del23, closed to the starting site as being important for EWS/FLI1 gene expression upon PI3K inhibition. So far little was known about possible transcriptional regulators of EWSR1, and only few factors were suggested and never validated [439]. Different *in silico* prediction and *in vivo* based programs identified transcription factors having their core binding region inside Del23; from these we then selected the ones known to be regulated by the PI3K pathway. Silencing of these factors pointed out Specificity Protein 1-SP1 as the main regulator of EWS/FLI1 gene expression. SP1 is a transcription factor described to bind to GC rich regions, and to be under the regulation of PI3K pathway through phosphorylation [440-443]. Treatment with Mithramycin, a competitor of SP1 for DNA binding, is already known to reduce EWS/FLI1 activity [294], thus confirming our findings. Silencing of PI3K subunits provoked similar effects to SP1 knock down and BEZ235 treatment; indeed EWS/FLI1 gene expression was reduced.

It is still not clear how PI3K regulates SP1, but we demonstrated that upon inhibition of the pathway, SP1 levels were reduced in the cells. In addition, to link SP1 to the region identified by the promoter study, we performed ChIP and Electrophoretic Mobility Shift Assay-EMSA to validate the binding both under *in vivo* and *in vitro* conditions.

Furthermore, we combined BEZ235 treatment with SP1 silencing and we observed a further downregulation of the fusion protein level.

Research with new dual PI3K/mTOR inhibitors is needed and more combinations have to be tested in Ewing tumour; nowadays a single drug treatment is not sufficient to defeat and prevent recurrence in ES patients. BEZ235 despite its promising results *in vitro* is no longer under study due to toxicity in clinical trials; a new compound also from Novartis (BKM120) has been tested in soft tissues sarcoma with promising results and therefore entered clinical trials [444, 445]. BKM120 indeed is a strong PI3K inhibitor, but its effect on Ewing cells unfortunately is not comparable to the ones induced by BEZ235 (data not shown).

Nevertheless, our data provide first insights on the transcriptional regulation of EWS/FLI1 fusion protein and we suggested a molecular mechanism for the known sensitivity of ES cell lines to PI3K inhibitors.

Since recent publications revealed also an important role of epigenetic modifiers in Ewing sarcoma [354, 446], we decided to focus on inhibition of epigenetic modifiers as new targeted approach. Epigenetic modifiers are responsible for gene expression or repression independently of the DNA sequence. Indeed epigenetic writers of this machinery add marks on the histones tails that are identified by the readers or will be removed by the erasers [348]. According to the type and position of the modification, gene transcription is activated or repressed.

EWS/FLI1 was described as capable of modifying the transition of chromatin from closed to open and to establish an active enhancer state [354, 446]. Indeed from an epigenome mapping study it emerged that EWS/FLI1 is responsible for a unique epigenetic signature [354].

Depletion of the fusion protein resulted in acetylated H3K27 in promoters, enhancers and superenhancers indicating that EWS/FLI1 is responsible for an open state of the chromatin. In this way it was possible to prove that the fusion protein is involved in epigenetic changes and through these we can identify more target genes regulated by EWS/FLI1.

Furthermore the fusion protein has been described to interact with the NuRD complex, involved in chromatin remodelling [150]. To the NuRD complex belong HDAC1 and 2, which are part of to the eraser class of epigenetic modifiers, and are responsible for removing acetyl groups from histones' tails. HDAC in ES is recruited by NKX2.2 repressor, an EWS/FLI1 target gene, and is responsible for NKX2.2 signature. Treatment with HDAC inhibitors was shown to suppress this signature and to reduce cell growth both *in vitro* and *in vivo* [144, 148, 359, 360]. Among EWS/FLI1 target genes there is also an epigenetic writer being part of the policomb repressor complex II, EZH2, a methyl transferase enzyme. Indeed EWS/FLI1 upregulates EZH2 expression that contributes to tumour development and metastasis *in vivo* [363].

Since new inhibitors of epigenetic reader were described to reduce tumour growth in different types of cancer [375, 376, 379-383], we decided to test for the first time reader blockers on Ewing cells, in collaboration with the group of Guenther Richter in Munich.

To this class belong BET proteins, in particular BRD proteins, that bind to specific lysine residues on histones tails [370], open the chromatin and recruit the transcriptional machinery [373] through direct interaction with positive transcription elongation factor b-P-TEF-b [447]. In addition BRD4 has been associated and described to regulate gene expression of the oncogene c-Myc [374, 378, 379] and of other genes also under the control of superenhancer regions [384]. Since oncogenes are often under the control of superenhancer regions or strong promoters [384, 385] and since EWS/FLI1 is an oncogene, we tested the BRD4 inhibitor JQ1 alone and in combination with BEZ235 on Ewing cells.

JQ1 is a small cell-permeable thieno-tiazolo-1, 4-diazepine, which blocks BRD4 by competing with the binding to acetylated lysines [367-370]. As mentioned before its efficacy in some types of sarcoma treatment was already described and nowadays this compound is of great interest for treatment of several tumours. This compound has been mainly described to reduce c-Myc expression, a gene involved in cell proliferation, transformation and vascular and hematopoietic development [448]. Therefore tumours having high expression of c-Myc are more sensitive to the treatment, indeed BRD4

blockage in Myc driven tumours such as neuroblastoma [449] or in medulloblastoma [450], or in high level c-Myc tumours as ovarian cancer [451] resulted in cell-cycle arrest and apoptosis.

We described for the first time that treatment with JQ1 reduces Ewing tumour growth and increase mice survival rate *in vivo* [452]. In addition it induces cell death in a dose and time dependent manner as well it inhibits cell proliferation and reduces colony formation capacity. Silencing of BRDs proteins was comparable to JQ1 treated samples signature, indicating the specificity of the compound.

Since Ewing cells present a high level of c-Myc expression [453] that is indirectly regulated by EWS/FLI1 [115, 153], we thought that one possible mode of action could be through c-Myc downregulation. Surprisingly c-Myc expression was not affected by JQ1 treatment. These data were confirmed by a similar and parallel study [454]. Indeed paediatric sarcomas, Rhabdomyosarcoma and Ewing sarcoma are sensitive to JQ1 treatment, nevertheless Rhabdomyosarcoma cells growth correlates with c-Myc level, whereas this was not true for Ewing cells. In addition it was described that JQ1 affects tumour growth by inhibition of angiogenesis, necessary for tumour maintenance.

We demonstrated for the first time a direct correlation in Ewing sarcoma between BRD4 inhibition and EWS/FLI1. Surprisingly, the fusion protein level is reduced upon treatment with JQ1. Blockage of BRD4 affects EWS/FLI1 gene expression and therefore its activity on target genes. We assume that the effects observed *in vivo* are the result of this downregulation.

This would also explain the effect on vascularization, in fact VEGF is a well known target gene of EWS/FLI1 [161]. The same effect on EWS/FLI1 level has been observed also by another group [455].

Since both BEZ235 and JQ1 treatment were reducing EWS/FLI1 gene expression, we tested *in vitro* the effect of a combination of these compounds. The result was a synergistic effect on induction of cell death [452]. This might be explained by the fact that BET inhibitors sustain PI3K pathway inhibition and enhance tumour cell death [456]. Indeed

combination of BEZ235 and JQ1 reduces cell viability of 50% *in vitro*, whereas *in vivo* it induces tumour regression and apoptosis of metastatic breast cancer cells.

A similar combination has been tested also in osteosarcoma cells, in which treatment with BRD4 inhibitors and PI3K inhibitors reduced cell viability in a synergistic way [457]. JQ1 treatment alone *in vitro* is responsible of reducing cell growth in several osteosarcoma cell lines that present high c-Myc expression and surprisingly also in this case the effects observed in cell proliferation were not due to a reduction in c-Myc level. Nevertheless, combination of JQ1 with Rapamycin enhances the antiproliferative effects and promotes cell death in xenograft model.

We were able to identify for the first time two different compounds that are affecting Ewing sarcoma cell growth due to a regulation on EWS/FLI1 gene expression. Knowing how the fusion protein is regulated might be the key for a better understanding of the disease and might lead to new molecular targets. So far it is not known the exact mechanism by which JQ1 inhibits EWS/FLI1 gene expression or the region involved at the promoter. We hypothesize that EWS/FLI1, being an oncogene, might have a superenhancer region where different regulators, and among them also BRD4, can bind and drive its expression. Combination of standard chemotherapeutics with new compounds or different inhibitors is the key for a more efficient inhibition; JQ1 treatment is relatively new in ES field and combinations *in vivo* have never been tried. Since PI3K inhibitors and JQ1 treatment enhance cell death in Ewing *in vitro* and since it is known how the crosstalk works, it is promising to test it further in xenograft model.

Indeed if Ewing cells are sensitive to JQ1 treatment and PI3K inhibition, we would expect a synergistic effect and an enhanced reduction of tumour growth.

Furthermore JQ1 has also other targets and can influence several important genes; it would be relevant for a targeted therapy to know all targets and reduce side effects. Therefore more microarray studies are required and new relevant targets can be uncovered. New unpublished studies identified XIAP and CDK9 to be downregulated after JQ1 treatment. This downregulation might be one of the mode of action of the drug to induce cell death. To uncover new mechanisms to enhance JQ1 effects, more

investigation and combination in this direction are highly recommended. Indeed combination of XIAP inhibitors as Birinopant or CDK9 inhibitors might result in a synergistic effect on cell death.

Another way to increase the efficacy of this compound was obtain by the same group that developed JQ1, by associating to the small molecule compound a phtalimide and the new drug is named dBET1 [458]. In this way the E3 ubiquitin ligase complex is recruited and can promote BRD4 degradation. The addition of phtalimide to small molecules inhibitors is a further inhibition of their target since it gets degraded due to the binding of phtalimide to Cereblon-CRBN a component of a cullin-RING ubiquitin ligase-CRL complex. Unfortunately during preliminary studies in Ewing, treatment with dBET1 does not further decrease EWS/FLI1 level, perhaps because drug concentration and time point are both critical for the formation of the complex as well the expression in this cellular context of all complex's components. Nevertheless the modification of small molecules inhibitors with phalimide is a potent alternative to the compound itself and enhances the effects.

So far we described compounds capable of affecting EWS/FLI1 activity by influencing its gene expression. There are also other ways to target a transcription factor and we also investigated how posttranslational modifications as phosphorylation affect EWS/FLI1 activity.

As mention above, so far there is no direct EWS/FLI1 inhibitor; nevertheless alternative and indirect ways to block EWS/FLI1 have been investigated.

Toretsky's group identified a RNA helicase A as interactor of EWS/FLI1 and disruption of this interaction reduces EWS/FLI1 activity [344]. Always in the same direction, it is known that BRD4 interacts with CDK9 driving gene expression and new evidences showed that EWS/FLI1 might interact also with this complex (data not published). Therefore targeting possible cofactors necessary for its function might deregulate EWS/FLI1 activity lowering its tumorigenic potential. Studying and identifying other interactors is of vital importance to find an alternative and specific approach. Another way is to target posttranslational modifications that might confer an advantage to the fusion protein. Indeed the activity of

transcription factors is regulated by posttranslational modifications [386]. These can affect protein activity, stability, cellular localization, protein-protein or protein-DNA interactions.
EWS/FLI1 has been described to have posttranslational modifications and some of them have been characterized as being relevant for its function.
Methylation of EWS/FLI1 indeed affects its cellular localization [392]; acetylation instead increases its function, whereas glycosylation reduces it, by altering the capability of EWS/FLI1 to bind DNA [390]. Phosphorylation and dephosphorylation of proteins is a key regulator of signal transduction [428]; different sites of EWS/FLI1 have been described to be phosphorylated suggesting a relevance of this modification also in Ewing sarcoma [387-389]. Therefore we investigated further the role of phosphorylation of EWS/FLI1 order to unravel new insights on the disease.
We identified by mass spectrometry a novel phosphorylation site, S287, in unstimulated conditions and presents in the FLI1 part. To test the role of such modification we mutated the serine 287 to alanine, in order to disrupt the phospho site. As result we observed a reduction in cell proliferation and colony formation capacity. The same has been observed also *in vivo* where the tumour growth driven by mutant EWS/FLI1 was lowered. To explain these effects we tested the activity of the mutant fusion protein; this single mutation indeed is also responsible for a lower activity of EWS/FLI1. By EMSA we could show that the mutant has a lower affinity to DNA binding compared to the wt and by qRT-PCR we also demonstrated that target genes of EWS/FLI1 were not induced or repressed as by the wt.
To confirm our results, recently it was published that treatment with Englerin A, a natural molecule, reduces EWS/FLI1 activity by affecting its phosphorylation state [389]. Indeed we both observed in absence of phosphorylation a lower binding to DNA, a reduction in proliferation rate and a G1 arrest.
Taken together our data could prove that posttranslational modifications such as phosphorylation are important for EWS/FLI1 activity and targeting upstream pathway and relative kinases involved in such process could lead to new targets for therapy.

MS in our laboratory identified some protein kinases to interact with EWS/FLI1; small screening with siRNA targeting those kinases unfortunately did not pinpoint any relevant protein affecting phosphorylation state of EWS/FLI1.

Despites our results, some evidences already pointed out PKCs as putative kinases responsible for EWS/FLI1 phosphorylation. It is well known that PRKCB is a target gene of EWS/FLI1 and its depletion resembles EWS/FLI1 silencing [234]; we hypothesized that PKCβ is the kinase involved in the fusion protein phosphorylation thus generating a positive feedback loop. Silencing of PKCs reduces EWS/FLI1 phospho-serines, but the site of phosphorylation has not been characterized [389].

Even if transcription factors cannot be directly targeted, we described two different indirect ways to do it. It is very important that new strategies are developed in order to design a more specific therapy and to unravel all possible targets for this aggressive pathology.

7. Literature

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